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THE INFLUENCE OF SEROTONIN, D-LYSERGIC ACID DIETHYLAMIDE AND 2-BROM-LSD ON THE INCORPORATION OF [^{35}S]METHIONINE INTO BRAIN PROTEINS AND ON THE LEVEL OF ATP IN THE BRAIN*

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IN previous papers we have described a fall in the relative specific activity (RSA)† of the proteins of the central nervous system of the rabbit after the intracranial injection of serotonin and D-lysergic acid diethylamide (LSD) together with [^{35}S]methionine or [^{14}C]glycine (KRAWCZYNSKI, 1959*a* and *b*; KRAWCZYNSKI *et al.*, 1958).

When these drugs were administered to rats they produced excitation and hypersensitivity, leading to convulsions. In considering the mechanism of the action of serotonin and LSD on the central nervous system, the possibility must be considered that endogenous as well as exogenous serotonin may induce convulsions by causing the contraction of the blood vessels in the brain. During the convulsions ATP may be broken down, and its resynthesis may be partially prevented owing to brain ischaemia. The resulting deficiency in ATP in the brain may lead to a fall in the RSA of the proteins (ROSENGART and MASLOWA, 1957; SHAPOT, 1957).

Investigations were undertaken into the effect of functional activity of the CNS produced by prolonged swimming on the RSA of proteins isolated from the brain hemispheres, cerebellum, brain-stem, kidney and liver after the injection of [^{35}S] methionine (KRAWCZYNSKI, 1960). The changes in RSA were dependent on the duration of the experiment. It was also found that a fall in the RSA of the brain proteins was associated with a fall in the ATP content of the brain. The aim of the present work was to determine whether or not the fall in the RSA of brain proteins produced by serotonin and LSD was accompanied by a fall in the ATP content of the brain.

Additional experiments were carried out in which 2-brom-LSD (BOL) or serotonin together with BOL were administered. Although the difference in structure between LSD and BOL is small, there are great differences in their pharmacological actions *in vivo* and *in vitro* (CERLETTI and ROTHLIN 1955; GADDUM and VOGT, 1956). The most interesting difference is that BOL, unlike LSD, exerts no hallucinogenic effect in man. Both substances inhibit the action of serotonin on smooth muscles *in vitro*.

Observations on the effect of LSD and BOL on the protein metabolism in the brain might throw light on the mechanism of the hallucinogenic action of LSD.

* This work was supported by the Biochemical Committee of the Polish Academy of Sciences.

† Abbreviations used in this paper are LSD—D-Lysergic acid diethylamide; BOL—2-Brom-LSD; RSA—Relative specific activity.

METHODS

The experiments were conducted on white rats weighing 280–320 g in experimental groups containing 3–4 animals. Each experiment was repeated several times and the results analysed statistically. Serotonin (2·5 mg/kg), LSD (250 μg/kg) and BOL (250 μg/kg) were administered intracranially together with [^{35}S]methionine (4000 counts/min per g). The volume of the administered fluid was 0·1–0·2 ml. Three hours after the injection of [^{35}S]methionine, the animals were killed by decapitation, the brain removed and the brain proteins isolated as previously described. The soluble fraction was also prepared as before; the ATP content was estimated according to the method described by MESZKOWA and SEWERIN (1950).

The relative specific activity of the proteins were calculated as follows:

$$\text{RSA} = \frac{\text{counts/min per mg of protein}}{\text{counts/min per ml of soluble fraction}} \times 100$$

The ATP content is given as mg of $P_{7\,min}$/g of wet tissue.

RESULTS AND DISCUSSION

The changes in the RSA of the proteins and in the ATP content of the rat brain after the injection of [^{35}S]methionine, serotonin, LSD and BOL, and also the behaviour of the animals, is given in Table 1. The results obtained agree well with those described previously. The few discrepancies are probably attributable to species differences between the animals used now and those used in earlier experiments (rats as opposed to rabbits).

TABLE 1.—RELATIVE SPECIFIC ACTIVITY (RSA) OF PROTEINS ISOLATED FROM RAT BRAIN AND THE ATP CONTENT OF BRAIN TISSUE AFTER THE INJECTION OF [^{35}S]METHIONINE (4000 counts/min per g), SEROTONIN (2·5 mg/kg), LSD (250 μg/kg) and BOL (250 μg/kg)

[^{35}S]Methionine and the drugs were administered intracranially. The animals were killed by decapitation 3 hours after the injection of [^{35}S]methionine and of the drugs. Mean values ± S.D.

Group	RSA	Change of RSA as per cent of the control		ATP as mg P_7/g tissue	Change of ATP content as per cent of the control		Behaviour
		(%)	(P) $t_e - t_0$		(%)	(P) $t_e - t_0$	
Control	27·77 ±3·32	—	—	0·120 ±0·021	—	—	normal
BOL	20·68 ±2·88	−22·3	<0·01 0·231	0·066 ±0·003	−45·0	<0·01 3·631	normal
LSD	19·07 ±2·10	−29·6	<0·01 1·860	0·110 ±0·018	−8·4	>0·1 −1·562	convulsions
Serotonin + LSD	17·61 ±2·02	−33·3	<0·01 2·782	0·097 ±0·008	−19·2	<0·05 0·373	excitation
Serotonin	16·80 ±2·10	−37·0	<0·01 3·121	0·065 ±0·005	−45·9	<0·01 3·631	normal
Serotonin + BOL	16·11 ±2·05	−40·7	<0·01 3·432	0·156 ±0·023	+30·0	<0·02 0·499	convulsions

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The rats in the control groups injected with [^{35}S]methionine only and in the groups which received serotonin or BOL behaved normally. In the groups in which serotonin was administered together with LSD, excitation of the animals and hypersensitivity to external impulses were observed. When LSD, or serotonin together with BOL, were introduced the rats were initially excited and then rapidly went into convulsions which persisted for from 2 to 4 minutes. In some experiments a few animals died.

All the compounds investigated, when injected either together or separately, decreased the RSA of the brain proteins. They can be placed in the following order according to the intensity of their effects: BOL, LSD, serotonin + LSD, serotonin, serotonin + BOL.

The changes in the ATP content of brain tissue did not parallel the changes in the RSA of brain proteins, but there was a certain correlation between the ATP content and the behaviour of the animals. A pronounced fall in the ATP content of brain tissue was observed in those groups in which the rats behaved normally, while in groups in which the animals were excited or had convulsions the decrease in ATP was less significant. In one of these groups (serotonin + BOL) an increase in the ATP content was noted. These results suggest that the disturbances in the serotonin equilibrium induced by the intracranial injection of serotonin and its antagonists LSD and BOL, might be the direct cause of the decrease in the ATP content of the brain.

On the basis of earlier investigations, it can be assumed that there is a further fall in the ATP content after the convulsions induced by LSD, and by serotonin + BOL. The energy producing processes partially compensate for the lack of ATP (adaptation phase); in certain conditions they may cause an increase in its level. The existence of biochemical compensation and hypercompensation has also been found by other workers (CZAGOWIEC, 1959; JAKOWLEW, 1955).

It is surprising that the increase in the ATP content of the brain, which can be called an adaptive one, does not exert any effect on the rate of incorporation of amino acids into the brain proteins or cause an increase of their RSA. There are two possible explanations for this: (a) that the rate of incorporation of radioactive amino acids into brain proteins is independent of the ATP level in the brain. In this case the serotonin, LSD and BOL could be regarded as acting directly on the protein metabolism of the CNS and not by an inhibition of the processes of oxidative phosphorylation; (b) that the RSA of the proteins depends on stores of energy in the cells at the time of isotope administration or immediately after it.

The observed differences between the actions of LSD and BOL on the RSA of brain proteins, on the ATP content in the brain and on the behaviour of the rats, suggest that, despite the close relationship in their chemical structure, they may affect different metabolic processes (BURIAN *et al.*, 1958; COSTA and ZETLER, 1958*a* and *b*). This assumes that these drugs act primarily on the metabolism, and not on the circulation of the brain.

It is possible that there is a connection between the hallucinogenic effect of LSD in man and its convulsive effect in rats. It may be assumed that the small doses of LSD which cause hallucinations in man induce only very weak alterations in the metabolism of the CNS. Larger doses, as used in the experiments described here, profoundly alter brain metabolism and induce convulsions. It is of interest that, although BOL itself is without apparent action on the CNS, it is a convulsive agent of great potency in the rat when used together with serotonin.

SUMMARY

[^{35}S]methionine (4000 counts/min per g) was injected intracranially into white rats together with serotonin, LSD and BOL. After 3 hours the RSA of the brain proteins and the ATP content of brain tissue were estimated. All the compounds investigated decreased the RSA of proteins. The ATP content was greatly reduced in groups of animals given serotonin or BOL and in those groups in which no alterations in the behaviour of the animals were observed. In other groups (LSD, serotonin + LSD, serotonin + BOL) in which convulsions occurred, the fall in the ATP content of the brain was much smaller; in the last group the ATP content was higher than in the controls. The possible explanation of these processes is discussed.

Acknowledgements—This work was conducted with the technical assistance of Mrs. ST. WISNIOWSKA and Miss H. MAZIARCZYK. I am very grateful to Fa. SANDOZ for supplying the serotonin, BOL and LSD.

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THE QUANTITATIVE SEPARATION AND ESTIMATION OF GLYCOSPHINGOSIDES IN NERVOUS TISSUE, WITH ESPECIAL REFERENCE TO GANGLIOSIDES

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As recently as 1955, SPERRY drew attention to the lack of a precise micro-method for the estimation of cerebrosides. Most of the values for 'cerebroside' given in the literature before 1955 include gangliosides, as it was the hexose constituent of the mixed lipids which was determined and both cerebrosides and gangliosides contain hexose. SVENNERHOLM (1956*a*) and LONG and STAPLES (1959*a*) have since developed micro-methods for cerebrosides. KLENK and LANGERBEINS (1941), KLENK and RENNKAMP (1941) and KLENK and LEUPOLD (1944) have described methods for the preparation of gangliosides and cerebrosides from brain on the macro-scale. CHATAGNON and CHATAGNON (1955*a*, *b*) have reported macro-methods for the preparation of both cerebrosides and gangliosides, using modifications of the method of FOLCH (1951). RADIN, LAVIN and BROWN (1955) developed a micro-method for the determination of cerebrosides only. SVENNERHOLM 1956*a*, 1957) has also described a micro-method for the estimation of gangliosides and LONG and STAPLES (1959*b*) a micro-method for N-acetyl neuraminic acid (NANA),* which might be used as an index of gangliosides. UZMAN and RUMLEY (1958) have published figures for cerebrosides and gangliosides in mouse brain, using neuraminic acid as an indirect measure of ganglioside, but UZMAN himself (UZMAN and RUMLEY, 1958) places no confidence in the value of his determination of neuraminic acid as a method for the estimation of ganglioside. TINGEY (1959) has measured glycolipids and discusses the inherent weaknesses of the calculations which he uses. But the quantitative determination of gangliosides, especially in white matter of nervous tissue still presents difficulties (KLENK and LANGERBEINS, 1941). EDGAR (1956) gave an excellent review of the methods and difficulties encountered in the quantitative estimation of the glycosphingosides.

WEISS (1956) separated sphingolipids by adsorption chromatography on silicic acid columns. He estimated the hexose component of the glycosphingosides by a modification of the anthrone method (RADIN *et al.*, 1955). The method reported in this communication is a modification of that of WEISS (1956) which can separate as little as 40–60 mg of crude sphingolipid. Although this is not as sensitive a method as that of SVENNERHOLM (1956*a*, 1957) or LONG and STAPLES (1959*a*), it has the advantage of giving greater detail of the glycosphingoside pattern.

MATERIALS AND METHODS

Materials. N-acetyl neuraminic acid was a gift from Miss PATRICIA CARROLL of this unit; a sample of cerebroside was given by Dr. R. RODNIGHT, and hyaluronic acid by Dr. H. J. ROGERS; a

* NANA = N-acetyl neuraminic acid.

sample of cerebronic acid was obtained through Dr. D. B. SMITH from Dr. H.E. CARTER; silicic acid A. R. Mallinckrodt, (suitable for chromatographic analysis by the method of RAMSEY and PATTERSON, 1945) was used. Chloroform was washed with water to remove ethanol, dried over Na_2SO_4, distilled, and stabilized with 2% (v/v) methanol. A. R. Methanol was used. Ether and light petroleum were dried over sodium, without further purification.

Half the cerebrum of dogs and total brains of rats were employed.

Methods. The brain was frozen immediately after removal from the animal and kept frozen until used. WEISS's (1956) method has been modified by:

(a) freeze-drying of the brain tissue over alumina;
(b) preparation of the silicic acid; and
(c) the determination of hexoses.

The material was freeze-dried over alumina pellets to avoid at least a 10 per cent loss of cerebroside which occurs when wet tissue is dried by stirring with acetone.

The dried tissue was powdered with an agate pestle and mortar before extracting three times with dry light petroleum b.p. (40°–60°). The residue was then air-dried at room temperature, after which it was extracted three times with boiling ethanol–water 95:5, v/v). The three ethanolic extracts were combined and left in a cold room at +4° for 48 hr, during which time the crude sphingolipid precipitated from solution. The precipitate was filtered off and the clear filtrate evaporated to half its original volume and again left in the cold for a further 48 hr. The precipitate which formed under these conditions was again removed and combined with the first precipitate. This constitutes our 'crude sphingolipid.' On standing at 4° for a month, no further lipid was precipitated from the filtrate.

The silicic acid was ball-milled to 300 mesh (British Standards) and then activated by heating to 135° for 6 hr (SPORER and TRUEBLOOD, 1959) to increase the resolving power of the column. The silicic acid was mixed to a slurry with ether–chloroform (2:1, v/v) poured into the column, and then washed according to TRUEBLOOD and MALMBERG (1949).

The 'crude sphingolipid' was placed on a column of silicic acid in chloroform–methanol (10:1, v/v) and the fractions obtained by gradient elution were evaporated to dryness and analysed. The eluant from the column was collected in 17 ml fractions which were separately evaporated and weighed. The fractions comprising an individual peak were combined and weighed again. The glucose and galactose content of these pooled fractions was determined after hydrolysis in 2 N-HCl (MONTREUIL, BOULANGER and HOUCKE, 1953) by paper chromatography. After hydrolysis for 45 min in 2 N-HCl, all the hexose was split off from the cerebroside, but not from the ganglioside. Although all the galactose was split from the latter, hydrolysis for a further 45 min was necessary to release all the glucose. The hydrolysate was extracted with a 5% (v/v) solution of N-methyldioctylamine in chloroform (MCFARREN and MILLS, 1952) and appropriate amounts of supernatant fluid at pH 5·5 were spotted alternately with glucose and galactose standards on Whatman no. 1 paper and developed for 72 hr at 23° in *n*-propanol–ethylacetate–water (ALBON and GROSS, 1952). The chromatogram was sprayed with aniline hydrogen phthalate (PARTRIDGE, 1949) and heated at 100° for 4 min in a water-saturated atmosphere. The brown glucose and galactose spots were then cut out with an appropriate blank spot of paper and the colour eluted in 3·5 ml of 0·7 N-HCl in 80% ethanol (LELOIR, 1951) and read at 400 mμ. Graphs were plotted relating optical density (OD) to concentration of standard sugars, and OD to concentration of cerebroside or ganglioside, from which the percentage of hexose in the lipid fractions was calculated.

Quantitative hexose determinations were carried out only on the fractions a, b and c (see Fig. 2), whereas NANA, hexosamine and hexoses were estimated in fractions d, e and f. The hydrolysates of all six fractions were submitted to paper chromatography for qualitative amino acid determination. The NANA was determined by BIAL's reaction according to WERNER and ODIN (1952) and hexosamine according to SVENNERHOLM (1956*c*) and GARDELL (1953).

RESULTS

Investigation of the methods used

The residue obtained after extraction with 95% aqueous ethanol was extracted in a Soxhlet apparatus for a further 6 hr with 95% ethanol. The solvent was then evaporated to dryness, when no residue was found. The twice-extracted tissue was again

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dried and then extracted in a Soxhlet apparatus with methanol–chloroform (2:1, v/v) for 2 hr. This solvent also yielded no residue on evaporation. The light petroleum washings of the dried brain tissue were found, by analysis, to contain no NANA, and only a trace of hexosamine (0·02 per cent). It may therefore be concluded that no ganglioside is extracted by dry light petroleum which chiefly removes cholesterol.

TABLE 1.—THE REPRODUCIBILITY OF THE METHOD OF EXTRACTION OF CRUDE SPHINGOLIPID FROM BRAIN TISSUE

	Weanling rat		Adult rat	
Dry wt (g/100 g moist tissue)	15·9		22·6	
Wt of dry brain tissue extracted (g)	4·045,	4·317	2·388,	2·385
Crude sphingolipid obtained (mg)	63·6,	65·2	184,	182
Crude sphingolipid in dry brain tissue (g/100 g dry wt)	1·57,	1·51	7·70,	7·63

The reproducibility of the method was verified by preparing the sphingolipids from two samples of brains pooled from weanling rats and from two samples of adult rat brain tissue (Table 1).

The gradient elution described by WEISS (1956) was checked by taking density measurements of the eluate at 50 ml intervals (Fig. 1). The eluted lipids affect the

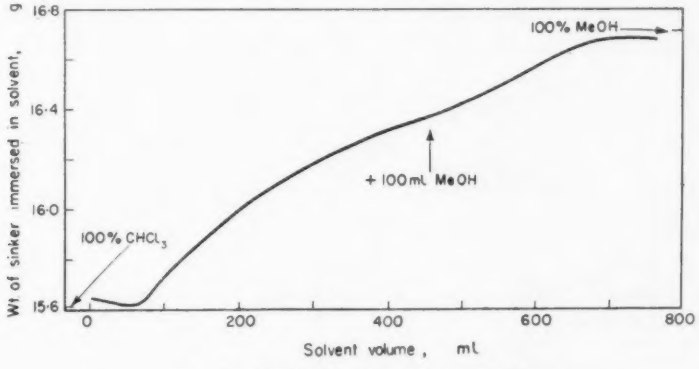

FIG. 1.—Showing gradient mixing of solvents.

specific gravity determinations so slightly that a smooth curve is found when plotting the weight of the sinker against volume.

To check that there were two water-soluble, ganglioside-like compounds, the crude sphingolipid was partitioned according to SVENNERHOLM (1956*a*). The aqueous phase containing the partitioned 'gangliosides' was removed, evaporated, and the residue, dissolved in 1 ml chloroform–methanol (10:1), placed on a column of silicic acid. The position and presence of the two 'gangliosides' was not altered by partitioning (Table 2).

To test the possibility of interference in the determination of NANA, BIAL's orcinol reaction was performed on a solution of ganglioside (obtained from the column) with and without the addition of a known quantity of NANA (Table 3). BIAL's reaction was also performed on a solution containing NANA and galactose in the amounts proportional to those found in ganglioside. The NANA absorption at 570 mμ was not increased by this quantity of galactose. Therefore, as the remainder of the ganglioside molecule does not interfere with the NANA determination, BIAL's reaction can be used directly on these separated lipids for the estimation of ganglioside.

TABLE 2.—N-ACETYLNEURAMINIC ACID AND HEXOSAMINE VALUES OF GANGLIOSIDE FRACTIONS AFTER PARTITIONING, COMPARED WITH THOSE SEPARATED ON A SILICIC ACID COLUMN

Sample	NANA (%)	Hexosamine (%)	NANA hexosamine (molar ratio)
Partitioned 'gangliosides'	12·5	3·64	2·0
1st eluted 'ganglioside'	4·3	Trace	—
2nd eluted 'ganglioside'	21·5	7·0	1·78

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Identification of eluted fractions

1. *Cerebrosides.* The methyl esters of the fatty acids of fractions b and c were prepared as follows: the phosphatide contaminants were first removed by shaking over-night at 37° with ethanolic-KOH (SCHMIDT *et al.*, 1946). The solution was neutralized with HCl and filtered. The residual cerebroside was hydrolysed with methanolic-sulphuric acid (CARTER *et al.*, 1947) to form the methylesters. Comparison of the infra-red spectra of these esters with that obtained by the diazo methane esterification

TABLE 3.—EFFECT OF THE REMAINDER OF THE GANGLIOSIDE MOLECULE ON THE DETERMINATION OF N-ACETYLNEURAMINIC ACID

Sample	Extinction at 570 mμ (1 cm cell)	Calculated NANA (μg)
Ganglioside	0·155	58·7
Ganglioside + 84·4 μg NANA	0·371	140·8
Estimated additional NANA	0·206	82·1
Recovery	97·2%	

of hydroxy-lignoceric (cerebronic) acid showed that fraction c contains hydroxy-lignoceric acid and is therefore, phrenosin. The selenite plate test (ROSENHEIM, 1914) was also performed on fractions b and c. This confirmed that fraction b was kerasin and fraction c, phrenosin.

2. *Gangliosides.* The ganglioside fraction e was treated according to SVENNERHOLM (1958) to confirm the presence of NANA. The ganglioside, eluted from a silicic acid column, was hydrolysed with 0·1 N-H_2SO_4 at 80° for 1 hr. The hydrolysate was cooled and then placed on a column of Dowex 2 and eluted with M-acetic acid–sodium acetate buffer. The eluate was collected and passed through a column of

Dowex 50. The eluate from this column was dried in vacuo over KOH. The residue was chromatographed in butanol–propanol–0·1 N-HCl (1:2:1, v/v) overnight and sprayed with KLEVSTRAND and NORDAL'S (1950) reagent. The spot obtained was found to have the same R_F as a sample of synthetic NANA. Fraction e was the major ganglioside fraction and had a molar ratio of $\frac{\text{NANA}}{\text{hexosamine}} = 1{\cdot}75$.

Fraction d contains galactose, glucose, a small amount of galactosamine and sphingosine. With BIAL'S orcinol reagent, it gives two peaks: one at 570 mμ, which may be due to neuraminic acid; and another peak at 440 mμ, due to hexoses, which is greater than that at 570 mμ. This latter peak is destroyed by acid treatment, as is the peak at 570 mμ due to NANA in ganglioside preparations (LONG and STAPLES, 1959*b*). From these facts we consider fraction d to be a ganglioside-like substance.

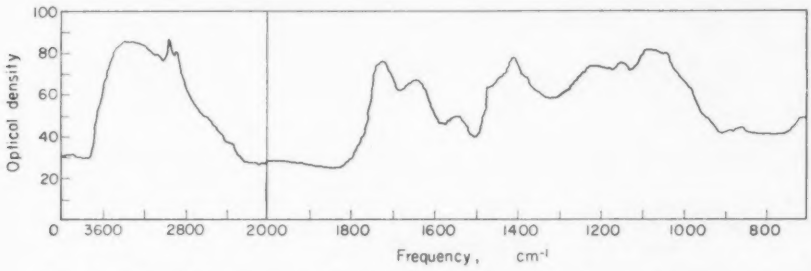

FIG. 2.—Infra-red spectrum of disputed ganglioside fraction d, separated on a column of silicic acid from a crude sphingolipid preparation from cerebral tissue.

Fraction f 'carnithin' gives a wave-length curve with BIAL'S orcinol reagent similar to that of SVENNERHOLM'S 'carnithin' (1957). Because of its reaction with orcinol, although the peak is not at 570 mμ, SVENNERHOLM reports it as being one of the substances interfering in the determination of ganglioside. We have found the wave-length curve with BIAL'S reagent to be the same as one given by a mixture of hexose and hyaluronic acid (another indication of its polysaccharide nature). With DISCHE'S (1947) carbazole reaction, 'carnithin' gives the brown colour due to hexoses, with no absorption peak at 530 mμ. This fraction is ninhydrin-positive.

Paper chromatography of eluted fractions. The hexose in fractions a, b and c was found to be galactose only. In the ganglioside fractions d and e, both glucose and galactose, as well as galactosamine, were found. No glucosamine was present. Sphingosine was found to be present in the first five fractions, but not in the sixth (f). Fractions c, d, e and f are contaminated with phospho-lipids. Part of fraction f overlaps the sphingomyelin which is eluted after 'carnithin'.

Infra-red spectra of eluted fractions. (KCl disc in a Perkin-Elmer double beam spectrophotometer). All fractions contained carbohydrate showing a strong doublet at 2860 cm^{-1} and 2930 cm^{-1} (—CH) and a strong band at 3300 cm^{-1} (—OH). All fractions (except 'carnithin', fraction f, which has a free NH_2 group, confirmed by the ninhydrin test) contained an amine linkage (doublet at 1640 and 1530). The main difference between fractions d and e (gangliosides) was that the former showed a more intense acid or ester band at 1725 and an intense absorption band at 1410 cm^{-1} (Figs. 2, 3, 4).

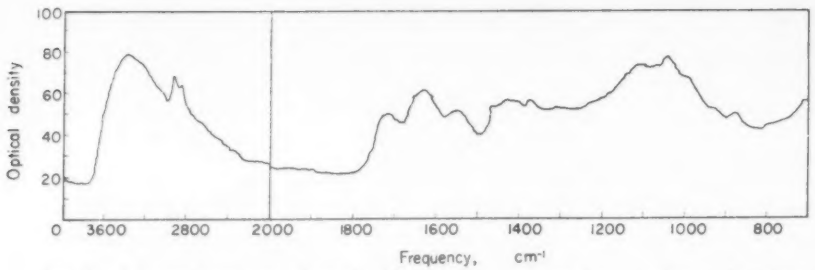

FIG. 3.—Infra-red spectrum of ganglioside fraction e, separated on a column of silicic acid from a crude sphingolipid preparation from cerebral tissue.

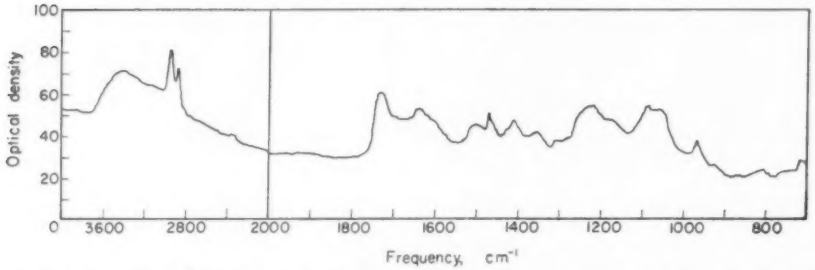

FIG. 4.—Infra-red spectrum of 'carnithin', fraction f, separated on a column of silicic acid from a crude sphingolipid preparation from cerebral tissue.

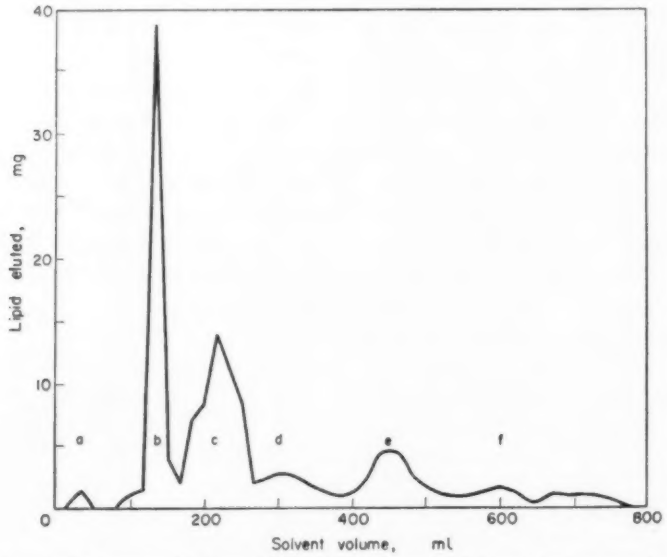

FIG. 5.—Elution pattern of glycosphingosides from cerebral tissue of a 2-year-old dog, on a silicic acid column. This pattern is typical of myelinated tissue.
Fraction a eluted with solvent front, C–M (10:1)
Fraction b—C–M (9:1)
Fraction c—C–M (2:1)
Fraction d—C–M (1:1)
Fraction e—C–M (1:2)
Fraction f—C–M (1:3)
where C = chloroform and M = methanol.

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Metachromasia. Fractions d, e and f all showed metachromasia with toluidine blue, in contrast with the cerebroside fractions which did not. The metachromasia in all three fractions, however, was not resistant to treatment with ethanol. This indicated that fraction f may be a polysaccharide, but that it is not sulphated.

The glycosphingoside pattern of dog brain (Fig. 5 and Table 4). The typical pattern of myelinated brain tissue consists of the first unadsorbed glycosphingoside fraction, the two major cerebroside fractions, the two 'ganglioside' fractions and 'carnithin'.

TABLE 4.—THE RECOVERY OF HEXOSE IN FRACTIONS SEPARATED FROM TWO PORTIONS OF CRUDE SPHINGOLIPID FROM 2-YEAR-OLD DOG CEREBRUM ON SILICIC ACID COLUMNS

No. of fraction	Amount collected (mg)	Galactose in fraction (%)	Galactose in fraction (mg)	Galactose in fraction as % of total column
a	2·4; 2·4	1·7; 1·9	0·041; 0·046	0·24; 0·27
b	53·9; 53·0	20·5; 21·3	11·030; 11·290	66·6; 66·2
c	36·4; 41·2	8·9; 7·3	3·242; 3·100	19·6; 18·2
d	9·3; 10·9	3·0; 2·9	0·280; 0·307	1·7; 1·8
e	20·2; 21·7	10·9; 10·8	2·208; 2·340	13·3; 13·7
f	14·1; 14·7	too faint to estimate	— —	— —
Total	136·3; 143·9		16·801; 17·033	
Crude sphingolipid on top of column (mg)		147·9; 150·8		
Galactose applied to top of column (mg)		17·880; 18·240		
Recovery of galactose (%)		94·0; 93·5		

The hexose figures above, for ganglioside are lower than figures quoted by other authors. This may be due partly to contamination with phospholipid and partly to estimating the sugars by paper chromatography instead of by the orcinol method (MONTREUIL and SCHEPPLER, 1959).

In the case of a 4-day-old dog cerebrum, only fractions e and f were found; so that myelination (as evidenced by the presence of cerebrosides) has not yet begun. This confirms the histological picture at this age.

The ratio of NANA to hexosamine. This was determined in the ganglioside fraction e of rat and dog brain by the method described (Table 5) and found to be relatively constant.

DISCUSSION

According to WEISS (1956), his evidence suggests that his band I is kerasin and band II is phrenosin, and that band III is another, unknown glycosphingoside containing an acetylated hexosamine. From the selenite plate tests on the fractions eluted from the column, and infra-red spectra on the methyl esters of the fatty acids of the cerebrosides b and c, our fraction b appears to be kerasin, containing lignoceric acid and fraction c is phrenosin containing cerebronic acid (Table 6). The relative proportions of fractions a to b and b to c also support this, and agree with the work of KISHIMOTO and RADIN (1959). We have found no trace of the acetylated hexosamine in fraction c (WEISS's band III).

WEISS's band IV, which he reports as being a mixture of substances, reacting with orcinol and ninhydrin, has been separated into three fractions d, e and f. We have found two 'ganglioside' fractions, d and e, not three as stated originally (PAYNE and PLATT, 1958). The third (f) has been found to be 'carnithin' (SVENNERHOLM, 1957).

KLENK and LAUENSTEIN (1953), SVENNERHOLM (1956*b*, 1956*c*), BOGOCH (1957),

TABLE 5.—THE RELATIVE CONSTANCY OF THE MOLAR RATIO HEXOSAMINE IN THE GANGLIOSIDE FRACTION e

Animal	Age of animal	NANA (%)	Hexosamine (%)	NANA hexosamine (molar ratio)
Dog	2 years	18·8	6·29	1·73
Dog	9½ weeks	19·6	6·23	1·82
Dog	8 weeks	17·4	5·80	1·74
Dog	6 weeks	20·3	6·90	1·71
Rat	21 day	18·9	6·20	1·76
		μg NANA in 1 ml solution	μg Hexosamine in 1 ml solution	
Dog	4 day	11·6	38	1·76
Rat	17 day	41·6	13·5	1·78

TABLE 6.—IDENTITY OF FRACTIONS ELUTED FROM SILICIC ACID COLUMNS

Fractions from silicic acid	Our identification	WEISS (1956) identification
a	unadsorbed glycosphingoside	band I kerasin
b	kerasin	band II phrenosin
c	phrenosin	band III unknown glycosphingoside
d	'ganglioside-like' compound	band IV containing ganglioside
e	ganglioside	
f	'carnithin'	

CHATAGNON and CHATAGNON (1955*a*, *b*), CUMINGS, GOODWIN and CURZON (1959), give various figures for the carbohydrate content of their gangliosides. The greatest variation is in the hexosamine figures, with the result that the NANA:hexosamine ratio varies from author to author. Some of the variations may be due to the fact that some brains are more mature than others. SVENNERHOLM (1956*b*, *c*, 1957) gives two sets of figures for a ganglioside fraction from human brain, one sample being prepared

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from senile brains, where the galactosamine value differs by 4·8 per cent and the hexose figure by 4·6 per cent. We have found that the ganglioside fraction e always has a molar ratio of 1·75 for NANA:hexosamine (SVENNERHOLM, 1956*b*; BOGOCH, 1958). An explanation of the discrepancy between the results of some other authors and our own may be that when ganglioside is estimated in the upper phase of a partition system, this contains not one, but two 'gangliosides', which have quite different NANA: hexosamine ratio. SVENNERHOLM (1956*b*, 1957) considers that the variation of the NANA:hexosamine ratio with age may be due to the occurrence of two gangliosides; the work reported here supports this. LONG and STAPLES (1959*b*) report extra carbohydrate in the upper phase of a partition system which they attribute to water-soluble sugars and their derivatives. It is possible that this extra carbohydrate corresponds to the 'ganglioside-like' fraction d which we have separated from the major ganglioside fraction e, on columns. The reagent which is usually used for the determination of N-acetylneuraminic acid (BIAL'S orcinol) is not specific for NANA, but gives coloured compounds with all sugars forming furfural or furfural derivatives. Therefore it is important to remove substances other than ganglioside from the lipid extract before the colour reaction is performed. We have shown that simple partitioning and/or dialysis does not do this. No method other than that developed in the present investigation allows the quantitative removal of all interfering contaminants. SVENNERHOLM (1957) measures the extinction at two wave-lengths, thus eliminating the influence of the contaminants. The peak at 570 mμ, which is formed when fraction d reacts with orcinol, may or may not be due to NANA. We have however been unable to obtain evidence on this point owing to lack of sufficient material.

If hexosamine is estimated in a chloroform–methanol extract of nervous tissue, a high hexosamine figure is obtained due to 'carnithin'. Therefore it seems clear that hexosamine determination cannot give a reasonably accurate measure of ganglioside. EDGAR (1958) has recently stressed that "hexosamine should not be announced as a determination of ganglioside." We conclude that for the accurate determination of ganglioside, both NANA and hexosamine estimations should be performed, and a purer specimen than that obtained by simple partitioning must be used.

Comparing the infra-red absorption spectra of our fractions with those of SCHWARZ, RIGGS and GLICK (1952) and of THOMAS, AUSTIN and DAVIES (1954) of tissue homogenates of foetal or very young, and adult brain, our ganglioside fraction e (which is found predominantly in grey matter in the brain) and 'carnithin' fraction f correspond to those of foetal or very young nervous tissue. The cerebroside (fractions b and c) pattern is similar to that found in white matter, where the amount of ganglioside in cerebroside is about 1 in 40. THOMAS *et al.* (1954) find that the band at 1075 cm^{-1} in adult brain shifts to 1087 cm^{-1} and in some cases to 1053 cm^{-1} in young brain. In our fraction f ('carnithin') we find bands at 1080 cm^{-1} and 1050 cm^{-1} as opposed to the band at 1070 cm^{-1} in fractions a to d. THOMAS *et al.* (1954) also find that the band at 1408 cm^{-1} is relatively much stronger in grey matter than in white. We find this band in fractions d, e and f, but very weak, if not absent, in fractions a, b and c (cerebrosides). Our findings also agree with those of SCHWARZ *et al.* (1952) that foetal brain (which we would expect to contain gangliosides) has an absorption band at 1046 cm^{-1} to 1036 $cm.^{-1}$ absent in adult brain (dominated by myelin, of which cerebroside is one of the components).

From the above it appears that fraction d is not a cerebroside, and not a true

ganglioside; it may be an intermediate compound in the synthesis of cerebroside from the breakdown products of a ganglioside.

SUMMARY

A crude sphingolipid extract of nervous tissue has been resolved by chromatography on silicic acid into two cerebroside fractions, b and c; another galactose-containing fraction, a; two ganglioside fractions, d and e; and 'carnithin', (fraction f). The main ganglioside fraction, e, has been found to have a relatively constant NANA: hexosamine ratio, irrespective of age.

The infra-red spectra of our fractions have been compared with those published by SCHWARZ (1952) and THOMAS (1954). The spectra of the first three fractions correspond to those they obtain from adult brain homogenates; fractions d,e and f have absorption bands which are found also in the spectra of foetal and young brain homogenates.

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Addenda (21 December 1960).

(1) From preliminary gas chromatographic analyses (by Dr. A. CROSSLEY at Unilever Ltd., Cheshire), both fractions b and c contain a number of fatty acids of varying chain lengths; both contain lignoceric acid, but fraction b contains much more lignoceric acid than fraction c.

(2) The ganglioside fraction e (see Fig. 5) has now been separated into two ganglioside fractions with NANA content of 30.2% and 20.8% which are almost identical with the figures for gangliosides $G_{0\cdot5}$ and G_2 reported by KUHN (1958, "*Carbohydrate Chemistry of Substances of Biological Interest*" Edited by WOLFROM, Pergamon Press, London), and with hexosamine contents of 8.3% and 9.3% giving $\frac{\text{NANA}}{\text{hexosamine}}$ ratios of 3.6 and 2.2 respectively, similar to those examined by SVENNERHOLM (1957, *Acta Soc. Med. Uppsala* **62,** 1) from grey and white matter of nervous tissue.

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EFFECT OF CONVULSANTS ON BRAIN GLYCOGEN IN THE MOUSE*

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THE first reliable method for the measurement of glycogen in brain tissue was published by KERR (1936). He and his coworkers found that a profound insulin hypoglycemia reduced the brain glycogen level in dogs, cats, and rabbits (KERR and GHANTUS, 1936; KERR, HAMPEL and GHANTUS, 1937). This change was confirmed in dogs by CHESLER and HIMWICH (1944), in cats by OLSEN and KLEIN (1947*a*, *b*), and recently in rats by SCHILLER (1958).

KERR and ANTAKI (1937) studied effects of picrotoxin, pentylenetetrazol, and strychnine in rabbits. These convulsants did not induce measurable changes in the brain glycogen. In these experiments, however, the convulsive activity was suppressed by administration of an anaesthetic before the brain was frozen. LEPAGE (1946) reported that intense and prolonged pentylenetetrazol seizures reduced the brain glycogen in rats. KLEIN and OLSEN (1947) tested a series of convulsants on cats paralysed with dihydro-β-erythroidine and found a decrease in brain glycogen associated with cerebral stimulation. In a small series of similar experiments on dogs under morphine, GURDJIAN, WEBSTER and STONE (1947) found that pentylenetetrazol did not induce statistically significant changes in the glycogen level.

CHANCE and YAXLEY (1950) reported findings in the mouse which are in sharp contrast with all of those quoted above. These workers studied the effects of insulin and a number of other convulsing agents, all of which appeared to increase the brain glycogen above the normal level. The conclusion was stated that an increase of glycogen in the mouse brain is specifically related to the convulsive discharge. Further data in support of this thesis were presented by CHANCE (1951, 1953) and by CHANCE and WALKER (1953–54).

This discrepancy has never been resolved. Since the possibility of a species difference is apparent, further studies of brain glycogen in the mouse seemed desirable. The determination of glycogen has been improved and simplified by the application, after hydrolysis, of a specific enzymic method for glucose. The convulsants used were pentylenetetrazol, picrotoxin, and insulin. Pentobarbital was also tested in order to compare the effect of an anaesthetic with those of the excitatory agents.

METHODS

White mice weighing 20–25 g were used. Dosages of drugs were as follows: pentylenetetrazol, 40 mg/kg body wt subcutaneously; picrotoxin, 4 mg/kg intravenously; insulin, 120–130 units/kg

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subcutaneously; and pentobarbital sodium, 80–120 mg/kg intraperitoneally. After injection of a convulsant, the mouse was observed until a seizure began, and during the seizure the whole mouse was frozen by immersion in liquid air. Mice given pentobarbital were frozen 15 min after injection, at which time the righting reflexes were always absent. Control animals received no injections.

The brains were removed while frozen (cerebella not included), and were ground to a powder for analysis. Usually three brains were ground together to provide a sample of approximately 1 g. The glycogen was isolated by the method of KERR (1936) and washed once with hot methanol–chloroform mixture (4:1 v/v). It was dissolved in 5 ml N-HCl, stoppered loosely, and hydrolysed by heating for 2 hr at 100°. The solution was neutralized by the addition of 15 ml of 0·835 M-dibasic sodium phosphate, and was filtered through glass wool. Glucose was determined on 1 ml aliquots by the method of SAIFER and GERSTENFELD (1958), the incubation being allowed to proceed at room temperature (22–25°) for 75 min. Glycogen was calculated as 0·927 times the glucose value.

RESULTS AND DISCUSSION

The mean value of brain glycogen in the control animals was 53 mg/100 g wet tissue (Table 1). This is based on seven determinations representing 21 mice. The value is lower than the known levels in dogs, cats and rabbits. Application of the enzymic method to dog brain (cortex from three animals, frozen *in situ* under morphine) gave values of 136, 121, and 161 mg/100 g. These data are comparable to those reported by KERR and GHANTUS (1936) and by GURDJIAN, WEBSTER and STONE (1947).

TABLE 1.—GLYCOGEN IN BRAINS OF MICE*

	Control animals [mg/100 g]	Pentobarbital anesthesia [mg/100 g]	Pentylenetetrazol seizures [mg/100 g]	Picrotoxin seizures [mg/100 g]	Insulin hypoglycemia [mg/100 g]
	50	59	37	33	15†
	45	46	39	38	30†
	54	51	37	37	24†
	46	56	41	33	18†
	53	55	37	38	22‡
	65	57	37		26‡
	58	68	38		26‡
Mean	53	56	38	36	23
S.D.	±6·9	±6·8	±1·5	±2·5	±5·1

* Each value represents an analysis of the combined brains of two or three mice.
† Frozen during insulin convulsions.
‡ Frozen in deep 'shock' without convulsions.

Application of the enzymic method to a series of 12 rat brains (cerebella excluded) gave a mean value of 70·7 mg/100 g ± 8·0 (S.D.). The rats were of the Sprague-Dawley strain (Holtzman), body weight 160 to 175 g, and were frozen under pentobarbital. This value is comparable to the mean of 66·5 mg/100 g reported by SCHILLER (1958), but is lower than some to be found in the literature. The enzymic method employs glucose oxidase, which is specific for glucose. In KERR's (1936) method the reducing power of non-glucose substances remaining after yeast fermentation is subtracted from the total reducing power. KERR pointed out that, despite thorough washing, the glycogen precipitate contains small but variable amounts of cerebrosides and other contaminants. On hydrolysis these yield galactose and possibly other non-glucose

reducing substances. In the use of KERR's method, the correction for these substances by yeast fermentation is a troublesome detail, neglect of which sometimes results in glycogen values that are too high.

CHANCE and his coworkers found glycogen values for normal mouse brain which are higher than those reported here, even though their animals were decapitated and the brains removed quickly without freezing. A series of 26 mice gave a mean value of 73·7 mg/100 g for cerebral cortex, and still higher levels were found in the other parts of the brain (CHANCE and YAXLEY, 1950; CHANCE, 1953; CHANCE and WALKER, 1953–54). These workers used KERR's method. They performed tests indicating that the reducing material in some of their hydrolysates was 97 per cent fermentable, but their determinations were made on very small tissue specimens, and apparently it was not feasible to make corrections by fermentation of each individual hydrolysate. The possibility of interfering substances in these determinations must be considered.

Effects of drugs. Pentylenetetrazol induced seizures after a latent period of 4–8 min and picrotoxin after 19–30 min. In each series the brain glycogen decreased, the change being significant at the 1% level (Table 1). These results are in agreement with the findings of LEPAGE (1946) on rats and of KLEIN and OLSEN (1947) on cats, and do not confirm those of CHANCE and his coworkers on mice. Thus no species difference in the brain glycogen metabolism is established.

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Insulin also produced a significant decrease in the brain glycogen level. Convulsions occurred after 1–2 hours in 60 per cent of the animals. Those which did not have convulsions were in hypoglycemic 'shock' and were frozen 2 hours after injection. The values observed in 'shock' did not differ appreciably from those during insulin convulsions. It might appear that convulsive activity did not contribute to the decrease in glycogen. However, blood glucose levels were not determined. The animals which did not have seizures may have had a more profound hypoglycemia than the others since the average time of insulin action was longer. Furthermore, it has been shown that some parts of the brain are depressed electrically, while others are hyperactive, during insulin convulsions (VAN METER, OWENS and HIMWICH, 1958). The glycogen levels in hypoglycemia were lower than those in pentylenetetrazol or picrotoxin seizures. The observed decrease in glycogen during hypoglycemia is in agreement with the findings of others in dogs, cats, and rabbits. In large part it undoubtedly reflects utilization of glycogen in lieu of glucose normally supplied by the blood. The finding of increased brain glycogen during insulin convulsions (CHANCE, 1951) is not confirmed.

During pentobarbital anaesthesia of 6 to 10 min duration the mean level of brain glycogen did not differ significantly from the control value (Table 1). This result is in agreement with the findings of CHANCE and YAXLEY (1950) and of OLSEN and KLEIN (1947*b*).

SUMMARY

Brain glycogen was determined, after fixation in liquid air, by a method employing glucose oxidase. The normal level was lower in the mouse brain than in the rat brain (cerebella not included), and lower in the rat brain than in the cerebral cortex of the dog. The level in mouse brain was unaffected by pentobarbital anaesthesia. It was decreased in insulin hypoglycemia and during convulsions induced by pentylenetetrazol or picrotoxin. The claim that positive increments of brain glycogen are associated with convulsive activity in mice could not be confirmed.

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THE INTRACELLULAR DISTRIBUTION OF COPPER IN BRAIN*

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A PREVIOUS report (PORTER and FOLCH, 1957*a*) described the separation from brain tissue under copper-free conditions of three different copper-containing fractions: Fraction I, extracted from the fresh tissue with 0·1 M-acetate buffer pH 4·5 (or with water or with 0·1 M-bicarbonate buffer pH 8·2); Fraction II, obtained by subsequent extraction of the tissue residue with water at pH 3·5 and vanishing ionic strength; and the residual Fraction III. Cerebrocuprein I, an essentially homogeneous brain protein containing 0·3% copper (PORTER and FOLCH, 1957*b*), has been isolated both from bovine Fraction I and from normal human brain (PORTER and AINSWORTH, 1959) and shown to bind its copper in a form which does not react directly with sodium diethyldithiocarbamate (PORTER and AINSWORTH, 1958). Attempts to isolate the brain copper proteins in Fractions II and III, however, have been unsuccessful. It seemed possible that the separation of particulate fractions by differential centrifugation might provide a more favourable starting point for the isolation of Fraction II and III copper proteins. The present paper describes the distribution of copper between cellular particulate fractions obtained from bovine cerebral cortex together with evidence that the major portion of the copper in the mitochondrial fraction from brain is in the form previously designated as Fraction III.

METHODS

Precautions to avoid contamination with extraneous copper, the general conditions of fractionation procedures, and the methods used for copper analysis and for preparation of cerebral cortex from fresh bovine brain were all identical with those employed in previous work (PORTER and FOLCH, 1957*a*; PORTER, 1959). The time between death of the animal and homogenization of cortex was estimated as about 3 hours. Measurements of pH were made with a Beckman model zeromatic pH meter.

Particulate fractions were prepared from bovine cerebral cortex by Procedure II of BRODY and BAIN (1952) with some modifications. Because of the large quantities (about 110 g) of tissue required in order to obtain sufficient material for copper analysis and for further fractionation of individual particulate fractions, homogenization was carried out by grinding for about 15 min in a mortar which had been precooled to −13°, followed by suspension with mechanical stirring in 9 vols. of 0·25 M-sucrose which had been adjusted to pH 8·5 with Na_2HPO_4. Centrifugations of homogenate and successive supernatant fluids were at 800 **g** for 10 min to separate residue R_1, at 1500 **g** for 10 min to separate residue R_2, at 13,400 **g** for 15 min to separate residue R_3 and at 40,200 **g** for 60 min to separate residue R_4 from the final supernatant fraction S. Supernatant fluids were decanted, any poorly

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sedimented intermediate layer being left with the residue, and the copper and solid contents of the residues were corrected for known volumes of supernatant fluid thus left in residual fractions. The composition of the fractions was checked by microscopic examination* after staining with Janus Green B. Some of the fractions were washed as follows: R_1 was reground in a mortar, resuspended in 2·5 volumes of sucrose and recentrifuged at the previously employed speeds to prepare washed R_1 plus R_2 and R_3 from the washings; R_2 was washed three times with 2·5 volumes of sucrose, and R_3 prepared from the combined washings; R_3 after freezing and thawing, was washed three or four times by resuspension in about 4 vols. of redistilled water and centrifugation at 40,200 **g**. Duplicate preparations of each fraction in amounts corresponding to 8 or more grams of fresh tissue were made from each homogenate and analysed for copper and dry weight after washing with acetone added to a final concentration of 75% (v/v). Supernatant fractions were brought to pH 5·2 before addition of acetone.

RESULTS

Distribution of copper and of solids between the particulate fractions. Microscopic examination confirmed that the predominant cellular element in each of the original fractions was as indicated in Table 1. The mitochondrial fractions appeared to be essentially free of intact nuclei and the microsomal fractions appeared to be free of recognizable mitochondria. Copper recovered in the sum of the individual fractions averaged 105 per cent of the total copper in the fresh tissue from which the fractions had been obtained; this result suggests that contamination with extraneous copper was of minimal degree.

The supernatant fractions contained 47 per cent of the total copper recovered in experiments A, B, and C. The combined mitochondrial fractions contained 31 per cent of the total copper recovered in experiments B and C. Washed and combined nuclear fractions contained 10 per cent and microsomal fractions 6 per cent, of the total recovered copper. Combined mitochondrial fractions accounted for 50 per cent of the total recovered solids. Microsomal fractions and combined washed nuclear fractions each contained less than 10 per cent of the total recovered solids.

Extraction of mitochondrial fractions at pH 3·5 and vanishing ionic strength. Mitochondrial fractions retained their copper content after freezing and thawing followed by washing four times with redistilled water. Two washed mitochondrial preparations were extracted with water after addition of 0·02 N-HCl to pH 3·5 and vanishing ionic strength under conditions similar to those employed for the previously described extraction of Fraction II copper proteins (PORTER and FOLCH, 1957*a*) from the tissue residue after extraction of Fraction I. The pH 3·5 extracts contained 0·19 μg and 0·21 μg of copper per g of fresh tissue or 24 per cent and 22 per cent of the starting mitochondrial copper. The insoluble residues contained 0·92 μg and 0·90 μg per g of fresh tissue or 116 per cent and 96 per cent of the starting mitochondrial copper. A third preparation was extracted at pH 3·1. This extract contained 0·25 μg of copper per g of fresh tissue or 39 per cent of the starting mitochondrial copper and the insoluble residue contained 0·46 μg of copper per g of fresh tissue or 72 per cent of the starting mitochondrial copper. Although some contamination with extraneous copper evidently occurred during these further fractionations of mitochondrial material, the results nevertheless suggest that the major portion of the mitochondrial copper of brain is in a form which remains insoluble at pH 3·5 and vanishing ionic strength, i.e. in the form previously designated as Fraction III (PORTER and FOLCH, 1957*a*)

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TABLE 1.—DISTRIBUTION OF COPPER AMONG PARTICULATE FRACTIONS FROM BOVINE CEREBRAL CORTEX*

Fraction		Expt.	Original fractions		With washings combined†	
			Copper	Solids	Copper	Solids
Total fresh tissue		A‡	2·28**			
		B‡	2·95**	118††		
		C‡	3·76**			
		mean	3·00			
Debris, whole cells	R_1	A	0·35	19·4		
		A	0·37	18·8		
		B	0·50	27·4	0·25	15·4
		B	0·37	25·5	0·15	14·4
		C	0·65	9·7	0·32	6·3
		C	0·66	10·3	0·39	5·9
		mean	0·48	18·5	0·28	10·5
Nuclei	R_2	A	0·16	19·3		
		A	0·26	21·3		
		B	0·29	21·4	0·30	19·4
		B	0·27	20·7	0·33	16·6
		C	0·24	13·5	0·39	6·7
		C	0·25	11·3	0·36	5·8
		mean	0·25	17·9	0·35	12·1
Mitochondria	R_3	A	0·53	46·4		
		A	0·61	48·3		
		B	0·66	51·2	0·92	66·4
		B	0·61	47·8	0·98	61·8
		C	0·95	54·4	1·18	65·9
		C	0·93	54·9	1·26	68·6
		mean	0·72	50·5	1·09	65·7
Microsomes	R_4	A	0·16	11·1		
		A	0·18	12·4		
		B	0·19	8·2		
		B	0·17	8·1		
		C	0·23	13·7		
		C	0·26	13·5		
		mean	0·20	11·2		
Supernatant	S	A	1·30	30·9		
		A	1·22	31·0		
		B	1·49	29·7		
		B	1·38	26·3		
		C	1·73	37·6		
		C	1·70	36·0		
		mean	1·47	31·9		

* Copper expressed as μg/g of fresh tissue. Solids expressed as mg/g of fresh tissue.

† In these columns R_1 is washed R_1; R_2 is washed R_2 plus R_2 from washings of R_1; R_3 is R_3 plus R_3 from washings of R_1 and R_2.

‡ Brains used in experiments A and B were from steers slaughtered by exsanguination. After this material became unavailable, brains used in experiment C were obtained from cows slaughtered by concussion. Some of the difference in absolute values obtained may be due to this difference in starting material. Inclusion of data from experiment C does not alter the average proportion of copper recovered in original fractions by more than 2 per cent.

** Mean of 3 determinations. All other copper figures represent the mean of 2 determinations.

†† Mean acetone-insoluble solids. Total tissue solids were about 170 mg/g of fresh tissue.

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DISCUSSION

In contrast to most studies dealing with the separation of cellular particles, in which material from laboratory animals has been processed immediately after death, the quantities required in the present investigation necessitated the employment of material obtained from the slaughter-house with resultant possible increase in liability to post-mortem artifact. The fractions obtained under the present conditions of about 3 hours delay between death and homogenization appeared, however, on microscopic examination, to be valid representations of the cellular particles.

In this study, 50 per cent of the total solids recovered from bovine cerebral cortex was found in the combined mitochondrial fractions. This figure for the proportion of cellular material in the mitochondrial fraction is considerably higher than the 17–33 per cent reported for such tissues as liver and kidney (SCHNEIDER and HOGEBOOM, 1951) and also exceeds the 22–39 per cent indicated by the data of other investigators for whole brain (JORDAN and MARCH 1956; PETERSEN and SHOU, 1955; CLOUET and RICHTER, 1959). Compared to the latter, the higher value found in the present investigation may be attributed in part both to inclusion of mitochondria washed out of Fractions R_1 and R_2 (as opposed to studies which did not include washings from these fractions) and to the greater numbers of mitochondria in the neuronal perikaryon and dendrites (POPE and HESS, 1957) of grey matter as opposed to axons (and glial cells) of white matter. Since the nature and bulk of the starting material employed here rendered the experiments so prolonged as to practically preclude adequate washing of mitochondrial fractions before freezing, allowance must be made for appreciable quantities of microsomes remaining with the unwashed mitochondrial fractions. However, if it is assumed that about 50 per cent of the 'true microsomal fraction' was sedimented with the mitochondria,* the present results would still suggest that mitochondria contribute a relatively large proportion, possibly up to 40 per cent, of the total cell mass of cerebral grey matter. That brain might have a larger proportion of mitochondria relative to other tissues might be anticipated from its reported very high oxygen consumption (KETY and SCHMIDT, 1945).

In agreement with the present results, some other reports have described recoveries of about 10 per cent of the total material in nuclear (JORDAN and MARCH, 1956) or microsomal (CLOUET and RICHTER, 1959) fractions from brain. Such a figure for the contribution of nuclei does not seem unreasonably small when one considers that the entire cell bodies (including nuclei) of neurons and glia together have been estimated to contribute only 10 per cent of the total volume of human cerebral cortex (POPE, 1955). The low figure for the contribution of microsomes is less clearly in accord with histological data and should be increased to allow for microsomes remaining with the unwashed mitochondria. However, a relatively low microsomal contribution in brain may be reconciled with histological findings, at least to some extent, by the above volume estimate plus the observations that Nissl substance (considered to represent microsomes) is absent in axons and is less abundant in dendrites than in the neuronal perikaryon (PALAY, 1956); these considerations suggest that a major portion of the microsomes may be in structures comprising only a small part of the volume of cerebral cortex.

* If the rate of incorporation of radioactively labelled amino acids is taken as a functional indication of microsomal distribution, the finding (CLOUET and RICHTER, 1959) that specific activity in microsomal fractions was 5 times greater than in unwashed mitochondrial fractions would suggest that a smaller proportion of the microsomes may remain with unwashed mitochondrial fractions from brain.

The largest portion of the cellular copper of cerebral cortex, amounting to almost one-half of the total copper recovered, was found in the supernatant fraction. This fraction presumably contains cerebrocuprein I (PORTER and FOLCH, 1957*b*), the brain copper protein previously isolated from Fraction I, and possibly other copper proteins soluble at neutral pH. The proportion of copper recovered in the supernatant fractions is larger than that previously found in Fraction I from bovine brain (PORTER and FOLCH, 1957*a*). It is therefore possible that the supernatant fraction also contains some of the copper proteins previously found in Fraction II but the relation of Fraction II copper to intracellular structure remains uncertain at present.

A second major portion of the cellular copper was found in the combined mitochondrial fractions which accounted for almost one-third of the total, whereas not more than one-tenth of the recovered copper was found in either the nuclear or the microsomal fractions. Since, in agreement with the original results of BRODY and BAIN (1952), Fractions R_1 and R_2 contained considerable amounts of mitochondria, some of which might persist after washing, it is possible that mitochondria actually contain more than one-third of the total cellular copper and that in this tissue nuclei do not contain significant amounts of copper. The bulk of this copper in mitochondrial fractions was not extracted at pH 3·5 and vanishing ionic strength and therefore appears to be in the form previously designated as Fraction III (PORTER and FOLCH, 1957*a*). The reports (WAINIO *et al*, 1959; MACKLER and PENN, 1957) of copper in mitochondrial subfractions having cytochrome oxidase activity suggest that Fraction III copper in brain mitochondria may represent, at least in part, copper associated with cytochrome oxidase. The extent to which copper associated with cytochrome oxidase accounts for the total copper content of mitochondria of brain has not yet been determined.

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SUMMARY

Particulate cellular fractions have been prepared from bovine cerebral cortex by differential centrifugation, and the distribution of copper and solids has been determined.

1. Of the total copper recovered, 47 per cent was in the supernatant fraction, 31 per cent in the combined mitochondrial fractions, 10 per cent in combined nuclear fractions, and 6 per cent in the microsomal fraction.
2. The major portion of the copper in mitochondrial fractions from brain was not extracted with water (Fraction I) or by subsequent extraction at pH 3·5 and vanishing ionic strength (Fraction II) and therefore appears to represent copper in the form previously described as Fraction III.
3. Combined mitochondrial fractions appeared to account for a relatively large proportion, possibly up to 40 per cent, of the total solids of cerebral cortex.

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THE REGULATION OF γ-AMINOBUTYRIC ACID METABOLISM IN CEREBRAL CORTEX MITOCHONDRIA

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IN BRAIN there are two alternative pathways for the metabolism of α-ketoglutarate to succinate: a *direct* pathway via succinyl-coenzyme A, and a *shunt* pathway through glutamic acid, γ-aminobutyric acid and succinic semialdehyde successively (ROBERTS, 1960). The latter pathway, which is exclusively in gray matter areas (ALBERS and BRADY, 1959; LOWE *et al.*, 1958; SALVADOR and ALBERS, 1959), is probably restricted to neurons and localized primarily in the mitochondria thereof (KOVAL and ALBERS, personal communication; MCKHANN *et al.*, 1960). Recent experiments have indicated that both of these metabolic pathways are potentially capable of supporting cerebral oxidative metabolism (MCKHANN and TOWER, 1959), and that *in vitro*, at least, there appears to be a reciprocal relationship between the metabolic activities of the two pathways (MCKHANN *et al.*, 1960). In the studies reported here some of the factors which may govern this reciprocity have been investigated *in vitro* with cat cerebral cortex mitochondria.

MATERIALS AND METHODS

Stock adult cats were used. Animals were sacrificed by compression-decapitation, and the brain was rapidly removed. The cerebral cortex was sliced off manually, weighed and immediately homogenized in 0·25 M-sucrose at 0°. Mitochondria were prepared by a modification of the BRODY and BAIN (1952) procedure in which fractions II-R_1 and II-R_2 were brought down together by centrifugation at 755 g for 10 min, and fraction II-R_3 was obtained by centrifugation at 18,800 g for 15 min, the entire procedure being carried out at 0° with a model SS-4 Servall centrifuge (MCKHANN and TOWER, 1961*a*). The mitochondrial pellet (fraction II-R_3) was resuspended in 9 volumes of cold 0·25 M-sucrose, washed once, and resuspended in a volume of 0·25 M-sucrose equivalent to the original fresh tissue weight. Examination of these fractions by electron microscopy (WANKO, GAVIN and MCKHANN, unpublished) indicated that these modifications in procedure result in a mitochondrial fraction freer of myelin and cellular debris than that obtained by the procedure originally described by BRODY and BAIN (1952). The residual contamination with microsomal particles was not considered significant for the experiments reported here.

Incubations were carried out at 25° in a specially designed Warburg flask fitted with a rubber-sealed port which permitted injection of acid into the vessel after the desired incubation period (MCKHANN and TOWER, 1961*b*). The incubation medium was a standard phosphate–saline medium of the following composition (final concentrations) in 2·0 ml: 50 mM-KCl, 8 mM-$MgCl_2$, 40 mM-Na-phosphate buffer (pH 7·2), 2·5 mM-ATP† and mitochondria equivalent to 0·5 to 1·0 g of original tissue in 0·25 M-sucrose. Specific substrates and additions are indicated with the appropriate results. Oxidative phosphorylation was measured as outlined by HUNTER *et al.* (1959) with adenosine monophosphate (AMP) as the phosphate acceptor and omission of diphosphopyridine nucleotide (DPN),

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† Abbreviations used. Adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP; diphosphopyridine nucleotide, DPN; γ-aminobutyric acid, γABA.

cytochrome *c*, and Versene® from the incubation mixture. Phosphate was determined by the method of ERNSTER *et al.* (1950). $^{14}CO_2$ evolved from labelled substrates was collected in Hyamine,® placed in the vessel centre-wells, and counted in a Tri-Carb® liquid scintillation counter (PASSMAN *et al.*, 1956).

All preparations were obtained commercially. The [1-^{14}C] γ-aminobutyric acid was obtained from the Research Chemicals Department, Merck and Co. Ltd., Montreal, Canada. It was found to be radiochemically pure by 2-dimensional phenol–lutidine chromatography, radioautography and subsequent strip counting and ninhydrin spraying.

RESULTS

P/O ratios. Previous experiments have demonstrated that γ-aminobutyric acid and succinic semialdehyde can support cerebral oxidative metabolism (MCKHANN and TOWER, 1959). In the present study satisfactory P/O ratios were obtained (Table 1)

TABLE 1.—OXIDATIVE PHOSPHORYLATION BY CAT CEREBRAL CORTEX MITOCHONDRIA

Substrates	Incubation time (min)	Oxygen consumption (μatoms/flask)	Phosphate esterification (μM/flask)	P/O
30 mM γ-Aminobutyric acid + 0·4 mM α-Ketoglutarate	15	1·18	3·0	2·54
	30	2·72	6·5	2·39
30 mM γ-Aminobutyric acid + 4 mM α-Ketoglutarate	15	4·0	10·1	2·52
	30	6·32	12·5	1·98

Each set of values represents mean of 2 experiments.
Conditions as described by HUNTER *et al.* (1959) with AMP as phosphate acceptor and omission of DPN, cytochrome *c*, and Versene® from the incubation mixture.

when cerebral cortex mitochondria were incubated with γ-aminobutyric acid as substrate for both 15 and 30 min over a 10-fold range of α-ketoglutarate concentrations (from 0·4 to 4·0 mM).

α-Ketoglutarate concentration. In these experiments mitochondria were incubated with a constant concentration of [1-^{14}C]γ-aminobutyric acid (30 mM, sp. act. 1·2 mC/mM) and varying concentrations of α-ketoglutarate, ranging from 0 to 6·5 mM (Table 2). As the concentration of α-ketoglutarate was increased, the fraction of total oxidative metabolism being supported by γ-aminobutyric acid decreased (Fig. 1). It would appear that under these conditions, as the concentration of α-ketoglutarate rises, oxidative decarboxylation of α-ketoglutarate is favoured over transamination with γ-aminobutyrate.

Blocking agents. The integrity of the γ-aminobutyric acid pathway and metabolism through it are known to be dependent upon the presence of adequate levels of pyridoxal phosphate (KILLAM and BAIN, 1957; MCKHANN *et al.*, 1960; ROBERTS, 1960). No evidence for inadequate levels of pyridoxal phosphate under the above conditions (Fig. 1) was obtained, as judged by supplementation of the incubation medium with the coenzyme.

On the other hand arsenite (OCHOA, 1941) and NH_4Cl (MCKHANN and TOWER, 1961*a*) exert an inhibitory effect on α-ketoglutarate decarboxylation while γ-aminobutyric acid metabolism is relatively unaffected by these compounds. Oxygen consumption with 4·0 mM-α-ketoglutarate as sole substrate was decreased some 60 per cent by 0·5 mM-arsenite (Table 3). With 30 mM-γ-aminobutyric acid present, the increased utilization of γ-aminobutyrate in the presence of arsenite suggests that there

TABLE 2.—TYPICAL EXPERIMENT WITH CAT CEREBRAL CORTEX MITOCHONDRIA ON THE RELATIONSHIP OF γ-AMINOBUTYRIC ACID UTILIZATION TO α-KETOGLUTARATE CONCENTRATION
(μM/flask/30 min)

α-Ketoglutarate conc. (mM)	γ-Aminobutyric acid utilization	Oxygen uptake/3	-γABA/-O_2 ratio (×100)
0	0·040	0·352	11·4
0·1	0·072	0·456	15·8
0·2	0·066	0·480	13·7
0·4	0·078	0·763	10·3
0·8	0·081	0·950	8·5
1·6	0·096	1·26	7·6
3·25	0·053	1·46	3·6
6·5	0·030	1·18	2·5

Conditions: Incubation at 25° in total volume of 2·0 ml containing 50 mM KCl, 8 mM $MgCl_2$, 40 mM Na-phosphate buffer (pH 7·2), 2·5 mM ATP, 30 mM [1-^{14}C] γ-aminobutyric acid (sp. act. 1·2 mC/mM), plus α-ketoglutarate as specified, and mitochondria equivalent to 0·7 g of original tissue, in 0·25 M-sucrose. Oxygen uptake was measured manometrically. CO_2 and $^{14}CO_2$ were collected in centre-wells of vessels in 0·2 ml of Hyamine^R. Incubation was stopped by injection of 0·2 ml 3 N-HCl as described under Methods in the text. After 45 min additional incubation, the $^{14}CO_2$-containing Hyamine was transferred quantitatively to counting vials and counted in a liquid scintillation counter for calculation of γ-aminobutyric acid utilization.

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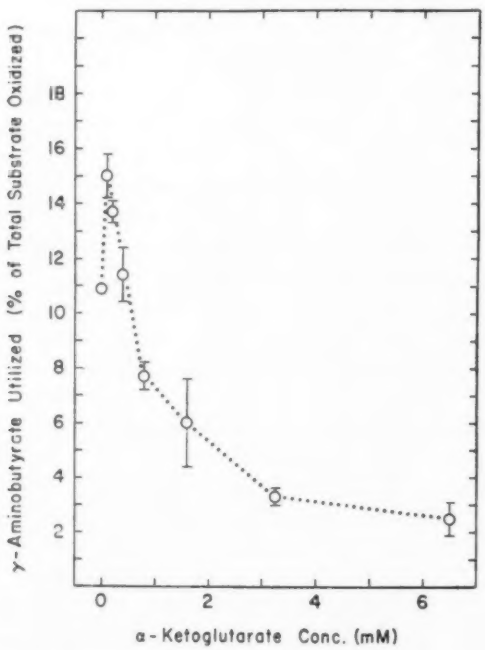

FIG. 1.—Effect of increasing α-ketoglutarate concentration (abscissa) on fraction of total substrate oxidized attributable to γ-aminobutyric acid utilization (ordinate) by cat cerebral cortex mitochondria incubated under conditions given in Table 2. Each point represents the mean of 4 or more experiments. Standard deviations indicated by the height of the vertical bars at each plotted point.

is no block of γ-aminobutyrate utilization and that higher concentrations of α-ketoglutarate *per se* do not decrease γ-aminobutyrate utilization.

The effects of 15 mM-NH_4Cl on γ-aminobutyrate utilization are illustrated in Table 4. At lower concentrations of α-ketoglutarate (0·1 to 0·5 mM) the ammonia present may compete with the transamination reaction by driving the glutamic dehydrogenase reaction in the direction of glutamic acid formation (STRECKER, 1957).

TABLE 3.—EFFECT OF 0·5 mM-K-ARSENITE ON UTILIZATION OF γ-AMINOBUTYRIC ACID BY CAT CEREBRAL CORTEX MITOCHONDRIA
(μM/flask/30 min)

Substrates	Arsenite	Oxygen uptake/3	γ-Aminobutyrate utilization	-γABA/-O_2 ratio (×100)
4 mM α-Ketoglutarate	0	1·53	—	—
	+	0·59	—	—
30 mM γ-Aminobutyric acid + 4 mM α-Ketoglutarate	0	1·65	0·093	5·6
	+	0·75	0·125	16·7
30 mM γ-Aminobutyric acid + 0·4 mM α-Ketoglutarate	0	0·77	0·112	14·6
	+	0·72	0·140	19·4

Conditions as in Table 2. Values are means of duplicate experiments.

TABLE 4.—EFFECT OF 15 mM-NH_4Cl ON UTILIZATION OF γ-AMINOBUTYRIC ACID BY CAT CEREBRAL CORTEX MITOCHONDRIA
(μM/flask/30 min)

Substrates	NH_4Cl	Oxygen uptake/3	γ-Aminobutyrate utilization	-γABA/-O_2 ratio (×100)
5 mM α-Ketoglutarate	0	0·47	—	—
	+	0·25	—	—
15 mM γ-Aminobutyric acid + 0·1 mM α-Ketoglutarate	0	0·83	0·09	10·8
	+	0·58	0·06	10·3
15 mM γ-Aminobutyric acid + 0·5 mM α-Ketoglutarate	0	0·7	—	—
	+	0·55	—	—
15 mM γ-Aminobutyric acid + 4 mM α-Ketoglutarate	0	0·97	0·057	5·9
	+	0·93	0·071	7·6

Conditions as in Table 2.

With higher levels of α-ketoglutarate (4·0 mM) no significant inhibition by NH_4Cl is evident.

Permeability. The permeability of mitochondria to various substrates varies considerably. One method of altering mitochondrial permeability is by allowing them to stand for a brief period (10 min) in hypotonic (0·075 M) sucrose (LEHNINGER, 1951). Studies of the effect of this pretreatment on the utilization of γ-aminobutyrate indicated (Table 5) that with 'swollen' mitochondria the depression of γ-aminobutyrate utilization by higher concentrations of α-ketoglutarate was not as great as with 'intact' mitochondria.

Effect of added DPN: KREBS (1959) has pointed out that when two substrates compete with each other as fuel for a metabolic system, this competition at the enzymic level may be a competition for DPN. Mitochondria are not normally permeable to oxidized DPN (BIRT and BARTLEY, 1960), but after pretreatment with 0·075 M-sucrose, DPN permeability is markedly enhanced (BIRT and BARTLEY, 1960; LEHNINGER, 1951).

TABLE 5.—EFFECT OF ALTERING PERMEABILITY OF CAT CEREBRAL CORTEX MITOCHONDRIA ON γ-AMINOBUTYRIC ACID UTILIZATION
(μM/flask/30 min)

30 mM γ-Aminobutyric acid plus α-Ketoglutarate conc. (mM)	Swelling	Oxygen uptake/3	γ-Aminobutyrate utilization	-γABA/-O_2 ratio (· 100)
0	0	0·23	0·030	13·0
	+	0·35	0·045	12·9
0·5	0	0·68	0·118	17·4
	+	0·65	0·130	20·0
5·0	0	1·25	0·048	3·8
	+	0·99	0·076	7·7

Values are means of duplicate experiments under each condition.
Mitochondria were pre-incubated for 10 min in 0·075 M-sucrose at 0°, then made up to original tissue weight with 0·25 M-sucrose and added to incubation flasks previously prepared with all remaining components (Table 2) and incubated at 25° for 30 min.

TABLE 6.—EFFECT OF ADDED 2·5 mM-DPN ON γ-AMINOBUTYRIC ACID UTILIZATION BY CAT CEREBRAL CORTEX MITOCHONDRIA
(Ratio ×100 of γ-aminobutyrate utilization to oxygen uptake)

Substrates	'Intact' mitochondria		'Swollen' mitochondria	
	Control	+ DPN	Control	+ DPN
30 mM γ-Aminobutyric acid, 1 mM α-Ketoglutarate	13·2	12·7	15·9	21·7 (±2·6)
30 mM γ-Aminobutyric acid, 4 mM α-Ketoglutarate	7·1	6·8	9·3	10·3 (±1·5)

Conditions as in Table 2 and Table 5. Values for 'intact' mitochondria are means of 2 experiments; for 'swollen' mitochondria means of 4 experiments. Owing to variations in metabolism of different mitochondrial preparations (Fig. 1), control results have been corrected to an empirical mean value and results with added DPN calculated from these so that standard deviations (in parenthesis) could be derived.

The effects of added DPN on γ-aminobutyric acid utilization are shown in Table 6. At lower concentrations of α-ketoglutarate (1·0 mM) a significant increase of γ-aminobutyric acid utilization was observed in 'swollen' mitochondria when 2·5 mM-DPN was added. No such enhancement was evident either at higher α-ketoglutarate concentrations or in 'intact' mitochondria. Similar trials with other cofactors (AMP, ADP, pyridoxal phosphate) were without effect.

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DISCUSSION

The data reported here (Table 2 and Fig. 1) provide further support for a reciprocal relationship between the fractions of total substrate metabolized by the direct (succinyl-coenzyme A) and γ-aminobutyric acid pathways in cerebral cortex. The effects of blocking agents (Tables 3 and 4) further illustrate this relationship and indicate that high concentrations of α-ketoglutarate *per se* are not a primary factor. Since DPN is known to be leached from mitochondrial preparations, especially in phosphate-buffered media (HUNTER *et al.*, 1959), the effect of DPN added to 'swollen' mitochondria (Table 6) at presumably more physiological concentrations of α-ketoglutarate is suggestive of a competition between the two pathways for available oxidized DPN and of the possibility that the DPN pool available to the γ-aminobutyrate pathway may be less tightly bound than that of the direct (succinyl-coenzyme A) pathway. The lack of effect of added DPN on 'intact' cerebral cortex mitochondria is consistent with similar observations previously reported for other tissues (BIRT and BARTLEY, 1960; LEHNINGER, 1951). The permeability effects (Table 5) suggest that swelling of the mitochondria may alter some factors concerned in the reciprocal relationship between the two pathways, at least at high concentrations of α-ketoglutarate, and perhaps in the latter case may also alter the response to added DPN.

The reciprocal relationship suggested by Fig. 1 qualitatively resembles that previously reported for incubated slices of cat cerebral cortex (MCKHANN *et al.*, 1960). In the latter study it was suggested that the type of relationship demonstrated in Fig. 1 might account for the variation of metabolic flux through the γ-aminobutyric acid pathway with time of incubation. From studies with incubated cat cerebral cortex slices it was calculated that over 40 per cent of the total substrate metabolized through the stage from α-ketoglutarate to succinate was metabolized via the γ-aminobutyrate pathway (MCKHANN *et al.*, 1960). Recent studies on mice (ALBERS *et al.*, 1961) and on a pyridoxine-dependent child (SOKOLOFF *et al.*, 1959) indicate that a comparable situation may obtain *in vivo*. In the present study with cat cerebral cortex mitochondria the maximal fraction referable to the γ-aminobutyric acid pathway was of the order of 20 per cent. When due allowance is made for differences in experimental design, for the problems of supplementing mitochondria, and for the fact that cerebral cortex mitochondria are derived from a heterogeneous cell population (of which only the neuronal complement possess the pathway in question), it is likely that the two sets of data are not too divergent.

All the available evidence (ALBERS *et al.*, 1961; MCKHANN and TOWER, 1959; MCKHANN *et al.*, 1960; ROBERTS, 1960; SOKOLOFF *et al.*, 1959) indicates that in cerebral neurons the γ-aminobutyric acid pathway represents a quantitatively and functionally important route in the basic sequence of neuronal oxidative metabolism. The present study on cortical mitochondria and a previous investigation with cortical slices (MCKHANN *et al.*, 1960) both point to a well-regulated integration of the activities of the two parallel metabolic pathways between α-ketoglutarate and succinate. Present data suggest that availability of oxidized DPN may be an important factor in this regulation. Whether such availability depends upon segregated pools of DPN or upon specific location of the respective pathways relative to a common DPN pool in the mitochondrial structure cannot at present be surmised. In view of the striking neuronal dysfunction associated with derangements of the γ-aminobutyric acid pathway (KILLAM and BAIN, 1957; MCKHANN *et al.*, 1960; ROBERTS, 1960; SOKOLOFF

et al., 1959; TOWER, 1960), the findings discussed here may have much more than a theoretical significance.

SUMMARY

Cat cerebral cortex mitochondria were used to study the previously suggested reciprocal relationship between substrate metabolism via succinyl-coenzyme A and via the γ-aminobutyric acid pathways at the α-ketoglutarate to succinate stage of cerebral oxidative metabolism.

With constant concentration of γ-aminobutyric acid, as the concentration of α-ketoglutarate was increased from 0 to 6·5 mM the fraction of total oxidative metabolism being supported by γ-aminobutyric acid decreased. This effect could not be attributed to high concentrations of α-ketoglutarate *per se*, as shown by the effects of 0·5 mM-K-arsenite and of 15 mM-NH_4Cl, which blocked α-ketoglutarate decarboxylation while γ-aminobutyric acid metabolism was relatively unaffected or significantly increased.

Altering mitochondrial permeability by brief pretreatment with hypotonic sucrose decreased the depressant effects of high α-ketoglutarate concentrations on γ-aminobutyric acid utilization. Addition of 2·5 mM-DPN to such 'swollen' mitochondria enhanced the utilization of γ-aminobutyric acid in the presence of low concentrations of α-ketoglutarate but exhibited no effects at high concentrations of the latter or with 'intact' mitochondria.

The role of DPN-availability in regulation of the reciprocal relationship of the two pathways and the quantitative significance of the γ-aminobutyric acid pathway for cerebral oxidative metabolism are discussed.

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ACTION OF INSULIN ON CARBOHYDRATE UPTAKE OF ISOLATED RAT SPINAL CORD

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IN A previous publication (RAFAELSEN, 1958) a technique of incubation of isolated rat spinal cord pieces in glucose-containing medium was reported. Using this technique, it was found that a definite uptake of glucose took place and that this glucose uptake was increased by insulin *in vitro*. Spinal cord pieces isolated from alloxan-diabetic rats had a decreased glucose uptake and insulin also increased the glucose uptake of these tissue samples.

The experiments reported here were concerned with a more detailed study of (i) the relation between insulin dose and glucose uptake *in vitro*; (ii) the effect of insulin on glucose uptake in the absence of oxygen; (iii) the effect of insulin on glucose uptake of homogenized rat spinal cord; and (iv) the effect of insulin on the uptake of some other sugars in isolated rat spinal cord pieces. Some experiments in which insulin was injected into the rat before isolation of the spinal cord instead of being added to the incubation medium, are also reported.

EXPERIMENTAL

Animals. Male and female albino rats (Wistar strain) weighing 80–100 g and fasted for 24 hr were used (water ad libitum). In any one experiment only rats of the same sex were used though there was no apparent difference between the results obtained with male and female rats.

Anaesthesia. The spinal cords were isolated from animals anaesthetized by inhalation of a mixture of 50% CO_2 and 50% O_2 for 2–3 min. In a few experiments, the animals were anaesthetized with ether, or unanaesthetized rats were decapitated, before removal of the spinal cord.

Blood sugar. In all experiments, blood samples were drawn immediately before sacrifice for determination of blood sugar values. Mean values of blood sugar in rats injected with insulin will be found in the appropriate Tables. Blood sugar values from rats injected with saline and from untreated rats were all in the range of 50–90 mg/100 ml.

Procedure. The method for isolation of rat spinal cord was as described earlier (RAFAELSEN, 1958). It was technically easier to isolate the spinal cord from rats weighing 80–100 g than from heavier rats. The preparation of the spinal cords from the 6–8 rats used for a single experiment lasted about one hour. The part between the cervical and the lumbar swelling was divided into three pieces (length of each piece about 10 mm, diameter some 3 mm, wet weight 50–100 mg). The spinal cord pieces were placed in ice-cold sugar-containing medium until all spinal cord pieces had been prepared. Each spinal cord piece was transferred to a 35 ml incubation flask containing 2 ml of medium and incubated separately. Aeration took place at 4° for 30 sec with a mixture of 95% O_2 and 5% CO_2. The flasks were incubated for 90 min at 37° in an apparatus with an oscillation rate of 84–88/min. The bicarbonate-buffered medium was as described by GEY and GEY (1936), except that magnesium was omitted. It contained per litre: 7·000 g NaCl; 0·370 g KCl; 2·270 g $NaHCO_3$; 0·335 g $CaCl_2,6H_2O$; 0·150 g Na_2HPO_4; and 0·030 g KH_2PO_4. The final concentrations of the individual ions were: 148 mM-Na^+, 5·2 mM-K^+, 1·5 mM-Ca^{++}, 128 mM-Cl^-, 27 mM-HCO_3^-, and 1·3 mM-PO_4^{---}. Unless otherwise indicated, the glucose concentration in the medium was 300 mg/100 ml. Other sugars used were

D-fructose, D-galactose, D-xylose, L-rhamnose, and D-arabinose, all in a concentration of 300 mg/100 ml.

In each experiment 8–12 control spinal cord pieces were incubated simultaneously with 8–12 spinal cord pieces under the influence of insulin. Mean values for carbohydrate uptake were calculated for control spinal cord pieces and for spinal cord pieces under the influence of insulin.

Two main types of experiment were performed. In one type, spinal cord pieces from untreated rats were incubated in medium without or in medium with insulin. Glucagon-free insulin containing 27 international units (i.u.) per mg was employed in concentrations of from 10^{-5} to 10^{-1} i.u./ml. Pieces of similar size were 'paired' for incubation in flasks without insulin and in flasks with insulin, but no attempt was made to pair spinal cord pieces belonging to the same animal.

In the other type of experiment, spinal cord pieces from rats given an intraperitoneal injection of either insulin or saline before sacrifice were incubated in medium without insulin. The volume injected was always 1 ml, and insulin was given in doses varying from 0·065 to 1·0 i.u./100 g body weight.

In a few experiments, spinal cord pieces were incubated under anaerobic conditions. A gas mixture containing 95% N_2 and 5% CO_2 was passed through the flasks for two periods of 120 sec; the flasks were shaken for 30 sec between the two periods. A homogenate of spinal cord tissue was obtained from rats weighing 250–400 g in order to get enough material using a limited number of animals. Homogenates were produced with a Potter-Elvehjem homogenizer (POTTER and ELVEHJEM, 1936). The amount of homogenate added to each flask corresponded to 50–100 mg wet weight and was consequently similar to the amount of tissue incubated in the experiments with structurally intact spinal cord tissue.

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Analytical methods

Carbohydrate uptake. Glucose, fructose, xylose, and arabinose were determined by the method of NELSON and SOMOGYI (SOMOGYI, 1952). Galactose and rhamnose were determined by the same method, but with a longer boiling period (HAFT, MIRSKY and PERISUTTI, 1953). After incubation, two samples of 0·100 ml were taken from the incubation medium in each flask for carbohydrate determinations. Samples were also withdrawn from control flasks aerated and incubated without tissue. Carbohydrate uptake was calculated from the difference between the carbohydrate concentration in the control flasks and that in the flasks containing spinal cord tissue and expressed as mg of carbohydrate/g of wet spinal cord per hr.

Wet and dry weight. Wet weights before and after incubation were determined in some of the experiments. Dry weights were determined in the same experiments after desiccation of the tissue pieces over P_2O_5 for 48 hr.

Histological examination. In some experiments the spinal cord pieces were examined histologically before and after incubation. The spinal cord pieces were placed in 10% aqueous formalin, embedded in paraffin, cut at 10 μ, and stained with haematoxylin–eosin, gallocyanin (Einarson), after VAN GIESON-HANSEN.

Statistical analysis. All values in the Tables are given $\pm$ S.E.M. (standard error of the estimated mean). The significance of the difference between means has been established by calculating 't'. *P*, the probability of difference being due to chance, was obtained from tables for 't' (FISHER and YATES, 1943).

RESULTS

Carbohydrate uptake

In the absence of insulin the mean glucose uptake of 249 control spinal cord pieces incubated in medium with 300 mg of glucose/100 ml was 3·24 $\pm$ 0·07 mg of glucose/g of wet spinal cord per hr. The day-to-day variation was of the same order of magnitude during the entire experimental period, but since it was rather large (50–100 per cent), control spinal cord pieces were incubated in every experiment as described above. Table 1 shows the glucose uptake of spinal cord pieces incubated in media with different glucose concentrations. It is seen that the glucose uptake was dependent on the glucose concentration in the medium so that a high glucose concentration led to a high glucose uptake by the spinal cord pieces.

Glucose uptake under anaerobic conditions did not differ significantly from that found under aerobic conditions (Table 2). The basal glucose uptake of spinal cord pieces and spinal cord homogenate was not directly compared, but the values for glucose uptake of spinal cord homogenates incubated without insulin (Table 7) were of the same order of magnitude as those for spinal cord pieces incubated without insulin (Table 3).

TABLE 1.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE FROM UNTREATED RATS INCUBATED IN MEDIA WITH DIFFERENT GLUCOSE CONCENTRATIONS

The data were obtained in a single experiment (Expt. no. 161)

Glucose conc. in medium (mg/100 ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet spinal cord/hr)	Significance of difference (*P*)
75	2·0 ± 0·14 (11)	
		<0·001
150	2·7 ± 0·09 (11)	
		<0·001
300	4·5 ± 0·26 (11)	
		<0·001
600	9·5 ± 0·39 (11)	

The number of spinal cord pieces is given in parentheses.

TABLE 2.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE FROM UNTREATED RATS INCUBATED AEROBICALLY OR ANAEROBICALLY

Expt. no.	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet spinal cord/hr)		Difference		Significance of difference (*P*)
	Incubated aerobically	Incubated anaerobically	(mg of glucose/g of wet spinal cord/hr)	(%)	
233	3·6 ± 0·21 (12)	3·5 ± 0·25 (12)	−0·1	−3	>0·05
234	4·6 ± 0·16 (12)	4·4 ± 0·25 (12)	−0·2	−4	>0·05
235	5·0 ± 0·11 (12)	4·8 ± 0·18 (11)	−0·2	−4	>0·05

The number of spinal cord pieces is given in parentheses.

The effect of insulin on glucose uptake of spinal cord pieces was studied in two ways. Table 3 shows the effect of insulin added to the incubation medium in concentrations varying from 10^{-5} to 10^{-1} i.u./ml. A statistically significant increase of glucose uptake was seen with all insulin concentrations above 10^{-5} i.u./ml in the incubation medium. The amount of extra glucose taken up in the presence of insulin increased with the insulin concentration. The mean increase of glucose with an insulin concentration of 10^{-4} i.u./ml in the medium was 40·1 ± 2·4 per cent (n: 64), whereas an insulin concentration of 10^{-1} i.u./ml in the medium led to an increase of 80·4 ± 5·6 per cent (n: 69) ($P < 0·001$). Most experiments were performed with a glucose concentration

of 300 mg/100 ml in the incubation medium, but it is seen from Table 3 that an insulin effect on the glucose uptake was also demonstrable with other glucose concentrations in the medium.

In further experiments the effect of insulin injected into the rats 15 to 120 minutes before removal of the spinal cord was investigated. Spinal cord pieces from rats

TABLE 3.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM

Expt. no.	Conc. of insulin in medium (i.u./ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet spinal cord/hr)		Difference (mg of glucose/g of wet spinal cord/hr)	Difference (%)	Significance of difference (*P*)
		Without insulin	With insulin			
193	10^{-5}	4·5 ± 0·14 (12)	4·6 ± 0·09 (12)	+0·1	+2	>0·05
164	10^{-5}	2·4 ± 0·14 (10)	2·3 ± 0·18 (10)	−0·1	−4	>0·05
79	10^{-4}	2·0 ± 0·29 (8)	3·1 ± 0·33 (8)	+1·1	+55	*<0·05*
163	10^{-4}	2·9 ± 0·16 (10)	3·7 ± 0·18 (10)	+0·8	+28	*<0·01*
181	10^{-4}	3·8 ± 0·18 (12)	4·9 ± 0·27 (12)	+1·1	+29	*<0·01*
182	10^{-4}	4·1 ± 0·18 (12)	4·9 ± 0·22 (12)	+0·8	+20	*<0·02*
183	10^{-4}	1·5 ± 0·32 (9)	2·5 ± 0·16 (9)	+1·0	+67	*<0·02*
184	10^{-4}	2·2 ± 0·16 (8)	2·8 ± 0·33 (9)	+0·6	+27	>0·05
192	10^{-4}	2·3 ± 0·19 (12)	3·1 + 0·19 (12)	+0·8	+35	*<0·01*
80	10^{-3}	2·3 ± 0·11 (8)	3·9 ± 0·36 (8)	+1·6	+70	*<0·01*
164	10^{-3}	2·4 ± 0·14 (10)	4·4 ± 0·19 (10)	+2·0	+83	*<0·001*
68	10^{-2}	4·5 ± 0·26 (12)	6·7 ± 0·44 (12)	+2·2	+49	*<0·001*
163	10^{-2}	2·9 ± 0·16 (10)	3·8 ± 0·17 (10)	+0·9	+31	*<0·01*
90	10^{-1}	3·2 ± 0·18 (7)	4·2 ± 0·26 (6)	+1·0	+31	*<0·02*
164	10^{-1}	2·4 ± 0·14 (10)	5·0 ± 0·14 (10)	+2·6	+108	*<0·001*
181	10^{-1}	3·8 ± 0·18 (12)	6·6 ± 0·29 (12)	+2·8	+74	*<0·001*
182	10^{-1}	4·1 ± 0·18 (12)	6·2 ± 0·17 (12)	+2·1	+51	*<0·001*
183	10^{-1}	1·5 ± 0·32 (9)	3·2 ± 0·21 (9)	+1·7	+113	*<0·001*
184	10^{-1}	2·2 ± 0·16 (8)	3·4 ± 0·32 (9)	+1·2	+55	*<0·01*
192	10^{-1}	2·3 ± 0·19 (12)	4·8 ± 0·18 (12)	+2·5	+109	*<0·001*
167*	10^{-1}	0·9 ± 0·08 (10)	1·6 ± 0·08 (10)	+0·7	+78	*<0·001*
165†	10^{-1}	5·2 ± 0·22 (10)	6·6 ± 0·25 (10)	+1·4	+27	*<0·01*

* glucose concentration in incubation medium: 150 mg/100 ml.
† glucose concentration in incubation medium: 600 mg/100 ml.
The number of spinal cord pieces is given in parentheses.

treated with insulin had a greater glucose uptake than spinal cord pieces from control rats treated with saline (Table 4). This effect was seen when the interval between the injection of insulin and excision of the spinal cord was as little as 15 mins. The increase was statistically significant and was higher with 1 i.u. of insulin than with lower doses.

Experiments were also performed with carbohydrates other than glucose in the incubation medium (Table 5). Insulin was without an effect on the uptake of D-arabinose, but led to a statistically significant increase of the uptake of D-fructose and D-galactose. The effect of insulin on the uptake of D-xylose and L-rhamnose was equivocal.

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TABLE 4.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE FROM CONTROL RATS TREATED WITH SALINE AND RATS TREATED WITH INSULIN

Expt. no.	Insulin injected (i.u./100 g body wt)	Time between injection and sacrifice (min)	Blood sugar at sacrifice Mean and range (mg/100 ml)	Glucose uptake Mean ± S.E.M. (mg of glusose/g of wet spinal cord/hr) Saline treated animals	Glucose uptake Mean ± S.E.M. (mg of glusose/g of wet spinal cord/hr) Insulin treated animals	Difference (mg of glucose/g of wet spinal cord/hr)	Difference (%)	Significance of difference (*P*)
145	0·065	60	42 (40–46)	3·6 ± 0·14 (9)	3·6 ± 0·24 (9)	0	0	—
145	0·125	90	31 (12–41)	3·6 ± 0·14 (9)	4·8 ± 0·24 (9)	+1·2	+33	*<0·001*
144	0·125	90	39 (18–59)	3·0 ± 0·20 (9)	4·5 ± 0·32 (9)	+1·5	+50	*<0·001*
143	0·25	90	24 (23–26)	3·0 ± 0·21 (11)	4·5 ± 0·25 (11)	+1·5	+50	*<0·001*
144	0·50	90	23 (15–29)	3·0 ± 0·20 (9)	4·1 ± 0·17 (9)	+1·1	+37	*<0·001*
141	1·0	15	44 (41–47)	2·5 ± 0·18 (11)	4·1 ± 0·21 (11)	+1·6	+65	*<0·001*
141	1·0	30	21 (15–31)	2·5 ± 0·18 (11)	4·4 ± 0·19 (11)	+1·9	+76	*<0·001*
140	1·0	45	21 (18–23)	3·3 ± 0·26 (11)	4·9 ± 0·20 (11)	+1·6	+48	*<0·001*
140	1·0	90	15 (10–23)	3·3 ± 0·26 (11)	7·6 ± 0·30 (11)	+4·3	+130	*<0·001*

The number of spinal cord pieces is given in parentheses.

TABLE 5.—UPTAKE OF VARIOUS SUGARS BY SPINAL CORD TISSUE FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM
Insulin concentration in medium: 10^{-1} i.u./ml.

Expt. no.	Sugar in medium	Sugar uptake Mean ± S.E.M. (mg of sugar/g of wet spinal cord/hr) Without insulin	With insulin	Difference (mg of sugar/g of wet spinal cord/hr)	(%)	Significance of difference (*P*)
196	D-fructose	2·7 ± 0·19 (12)	3·4 ± 0·15 (12)	+0·7	+26	*<0·02*
197	D-fructose	2·9 ± 0·14 (12)	3·3 ± 0·12 (12)	+0·4	+14	*<0·05*
187	D-galactose	2·8 ± 0·18 (12)	3·4 ± 0·21 (12)	+0·6	+21	*<0·05*
201	D-galactose	3·0 ± 0·28 (12)	3·8 ± 0·20 (12)	+0·8	+27	*<0·05*
199	D-xylose	2·6 ± 0·11 (12)	2·9 ± 0·11 (12)	+0·3	+12	>0·05
203	D-xylose	3·5 ± 0·17 (12)	4·0 ± 0·22 (12)	+0·5	+14	>0·05
185	L-rhamnose	4·0 ± 0·26 (12)	4·4 ± 0·37 (12)	+0·4	+10	>0·05
200	L-rhamnose	2·6 ± 0·16 (12)	3·0 ± 0·38 (12)	+0·4	+15	>0·05
194	D-arabinose	3·6 ± 0·21 (12)	3·5 ± 0·20 (12)	−0·1	−3	>0·05
198	D-arabinose	2·1 ± 0·14 (12)	2·0 ± 0·14 (12)	−0·1	−5	>0·05

The number of spinal cord pieces is given in parentheses.

TABLE 6.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE FROM UNTREATED RATS DURING ANAEROBIC INCUBATION WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM
Insulin concentration in medium: 10^{-1} i.u./ml.

Expt. no.	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet spinal cord/hr) Without insulin	With insulin	Difference (mg of glucose/g of wet spinal cord/hr)	(%)	Significance of difference (*P*)
229	4·4 ± 0·18 (12)	5·0 ± 0·21 (12)	+0·6	+14	>0·05
230	3·7 ± 0·14 (12)	4·1 ± 0·16 (12)	+0·4	+11	>0·05
231	3·7 ± 0·10 (9)	3·6 ± 0·11 (9)	−0·1	−3	>0·05
232	3·4 ± 0·14 (9)	3·7 ± 0·15 (9)	+0·3	+9	>0·05

The number of spinal cord pieces is given in parentheses.

Anaerobic conditions during incubation did not lead to any change in the amount of glucose taken up but insulin was without an effect on the glucose uptake of spinal cord pieces incubated anaerobically, even when added to the medium in a concentration of 10^{-1} i.u./ml (Table 6). No effect of insulin on glucose uptake of rat spinal cord homogenate was observed. Insulin 10^{-1} i.u./ml in the medium did not increase the glucose uptake significantly in any of the experiments performed (Table 7).

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TABLE 7.—GLUCOSE UPTAKE OF SPINAL CORD TISSUE HOMOGENATE FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM
Insulin concentration in medium: 10^{-1} i.u./ml.

Expt. no.	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet spinal cord/hr)		Difference		Significance of difference (P)
	Without insulin	With insulin	(mg of glucose/g of wet spinal cord/hr)	(%)	
105	1·8 ± 0·35 (10)	1·3 ± 0·41 (9)	−0·5	−31	>0·05
236	2·7 ± 0·31 (11)	3·0 ± 0·35 (11)	+0·3	+11	>0·05
237	2·9 ± 0·22 (12)	3·0 ± 0·33 (11)	+0·1	+3	>0·05

The number of spinal cord pieces is given in parentheses.

Solid matter content

In untreated rats a percentage dry weight of 31 (n: 24) was found in spinal cord pieces dried immediately after excision. In spinal cord pieces desiccated after soaking and incubated in medium without insulin the percentage dry weight was 27 (n: 199). The percentage dry weight after soaking and incubation was also 27 (n: 72) in spinal cord pieces from rats injected with insulin before sacrifice. With no loss of tissue fragments an increase in wet weight during soaking and incubation of 15 per cent was to be expected from the changes in percentage dry weight observed. Actually, an increase in wet weight of 4 per cent was found (n: 33), so the spinal cord pieces had suffered a mean loss of debris or tissue fragments amounting to about 10 per cent of the original wet weight.

Histological changes

Groups of spinal cord pieces were withdrawn for histological study at four stages: (1) immediately after excision; (2) after soaking in ice-cold buffer for 60 minutes; (3) after incubation at 37° for 15 minutes; and (4) after incubation for 90 minutes. Group 1 showed no pathological lesions (nerve cells well-defined, clearly visible rather coarse Nissl granules in the motor cells. No vacuoles were seen in the cytoplasm and dendrites were stained for only a short distance from the cell bodies). Group 2 showed small vacuoles in some of the larger motor cells, otherwise no changes from 1. In group 3 the motor cells were less well-defined, Nissl granules and cells as a whole being stained somewhat darker, but there were also cells with diminution of Nissl granules and somewhat vacuolated cytoplasm. Dendrites were stained for a longer distance from the perikaryon. Nuclei were often somewhat hazy, but with preserved nucleoli. In group 4 some cells had distended cytoplasm with vague limits and vacuoles. The cytoplasm of the motor cells was often diffusely stained with only rudiments of Nissl granules, but the cells as a whole were chromophile. Glial cells were often frayed at the edge.

DISCUSSION

The present investigation shows that insulin has a direct effect on the carbohydrate uptake of the isolated rat spinal cord. The glucose uptake increases with increasing insulin concentration in the medium and insulin has also an effect on the uptake of some other sugars, e.g. D-fructose and D-galactose. Insulin has no effect on

the glucose uptake of spinal cord pieces in the absence of oxygen, nor is the glucose uptake of rat spinal cord homogenate increased by insulin. It is often stated that insulin is without an effect on the central nervous system, but relatively few papers have been concerned with the direct action of insulin. In those investigations in which an effect has been observed, it has probably been the result of an indirect action of insulin, due to hypoglycemia, but the possibility of a direct effect of insulin on the central nervous system acting in combination with this cannot be excluded. The distinction between a direct and an indirect effect of insulin is very important (for a discussion see RAFAELSEN, 1961*b*).

Isolated rat spinal cord pieces seem structurally more intact than the samples from the central nervous system commonly used for *in vitro* studies. On the other hand, the surface area per unit weight is much smaller and the diameter much greater than is usually accepted for *in vitro* studies. The maximum permissible thickness for tissue slices if full oxygen saturation is to be obtained throughout the slice has been calculated to be about 0·4 mm in an atmosphere of oxygen (WARBURG, 1930; FIELD, 1948). Brain, liver, and kidney can be cut in slices to fulfill this demand, but, except for the first slice, the slices obtained have damaged surfaces. It is very difficult to slice muscle, but a rat hemidiaphragm from a 100 g rat has a mean thickness of 0·5 mm and this sample combines a large surface relative to weight (about 300 mm^2, mean weight 100 mg) with the possible advantages of good structural preservation: few cut cells and intact organ membranes over the larger part of the sample. In a search for a tissue sample from the central nervous system which could combine a large surface area relative to weight with good structural preservation, the rat spinal cord was chosen although it does not have as good a surface : weight ratio as the rat diaphragm. The surface area per unit weight of the spinal cord pieces is only 40 per cent of that of isolated rat diaphragm when the tissue samples are taken from a 100 g rat. The cut ends of the spinal cord pieces are disadvantages, but so are the cut edges of the isolated rat diaphragm.

The rat spinal cord pieces have a diameter of about 3 mm which means that full oxygenation in the interior of the tissue cylinder is not obtained. LARRABEE (1958) encountered a similar problem when measuring oxygen consumption of excised rat sympathetic ganglia and discussed the theoretical and practical implications of an 'anoxic core' in the sample. He found that some, but not all, of his samples had an anoxic core in solution equilibrated with 95% O_2 at 36°, but in spite of the anoxic core in some of the preparations the isolated rat superior sympathetic ganglion proved a useful preparation for incubation studies, and it was found that the oxygen and glucose uptake increased during repetitive stimulation (LARRABEE, 1958; HOROWICZ and LARRABEE, 1958). If a diameter of 0·5 mm represents the limit for full oxygen saturation it is even less likely that full glucose saturation takes place in the centre of rat spinal cord cylinders, and it may well be that the core takes part to only a limited degree, or not at all, in the phenomena observed during incubation.

Isolated tissue samples swell, i.e. take up fluid from the medium during soaking and incubation, but samples from different organ systems vary quantitatively very much in this respect. Isolated spinal cord pieces and brain slices were compared. The increase in wet weight during incubation is equal to the swelling of the sample minus the loss of debris or tissue fragments which the sample suffers during incubation. It was calculated that spinal cord pieces swelled about 15 per cent whereas both first and

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second brain slices swelled about 60 per cent during soaking and incubation in glucose-containing medium without insulin.

The histological investigation was included in order to see whether a morphological change took place in isolated rat spinal cord between isolation of the tissue and the end of incubation. It is evident that isolated tissues are dying tissues, but the results reported in this paper and in a following one (RAFAELSEN and CLAUSEN, 1961) show that the spinal cord pieces are metabolically active. The changes observed were those usually seen post mortem and they were of moderate intensity. Incubation for 15 or 90 minutes did not cause gross changes in the appearance of the nerve cells with any of the stains used. Thus the rat spinal cord piece is a relatively stable sample from a morphological point of view during an incubation period of 90 minutes, but the moderate morphological changes of the nerve cells do not regress during incubation in spite of the biochemical activity of the sample.

The glucose uptake of spinal cord pieces increased when higher glucose concentrations were used in the medium. Calculation from the figures given in Table I suggests that the relation between glucose concentration in the medium and glucose uptake is of the log.normal type. In perfusion experiments a value of 7·7 mg of glucose/g of wet spinal cord per hr was obtained by TSCHIRGI *et al.* (1949). In these experiments the rat spinal cord was supported on the vertebral bodies fixed in plaster and the glucose concentration in the perfusion fluid was 100 mg/100 ml. Under these conditions reflex activity was maintained for hours. The glucose-offer to the tissue must be very different in perfusion studies and in incubation studies. In the latter type of experiments the diffusion of glucose to the separated samples must occur from the surfaces without facilitation by the blood capillaries, but if the glucose uptake is compared on the basis of the glucose concentration in perfusion fluid and incubation medium it is evident that the glucose uptake was much higher in the perfusion studies of TSCHIRGI *et al.* (1949) than in the present studies. Similar results have been obtained in experiments with cerebral tissues where the respiratory rate of unstimulated brain slices is maximally 50 per cent of the values obtained *in vivo* (MCILWAIN, 1959).

Glucose uptake *in vitro* of different isolated tissues vary considerably both in the absence and presence of insulin. This applies not only to results from experiments with different tissues, but also to the results obtained by different workers with the same type of tissue. It is important to realize that minor modifications in the experimental conditions, e.g. the composition of the incubation medium, may modify the results to a great extent (STADIE and ZAPP, 1947; KRAHL and PARK, 1948; DOMONKOS and HUSZAK, 1959; ROGERS, 1958; BELOFF-CHAIN *et al.*, 1953, 1955; VILLEE, DEANE and HASTINGS, 1949; TAKAGAKI and TSUKADA, 1957; GEMMILL and HAMMAN, 1941; BROWN *et al.*, 1952). With rat diaphragm several workers have found a dose response of insulin on glucose uptake (e.g. KRAHL and PARK, 1948; RANDLE and SMITH, 1958). In rat spinal cord experiments a parallel phenomenon was observed. If the smallest insulin concentration capable of increasing the glucose uptake *in vitro* is taken as an expression of the sensitivity of the tissue to insulin, it is seen that isolated rat diaphragm and isolated rat spinal cord pieces resemble each other in this respect. In rat diaphragm an effect of insulin on the glucose uptake has been demonstrated with insulin concentrations as low as 10^{-4} to 10^{-5} i.u./ml in the medium (VALLANCE-OWEN and HURLOCK, 1954) and in rat spinal cord experiments 10^{-4} i.u./ml insulin was found to be the smallest concentration which increased the glucose uptake.

It is well known that the stereochemical configuration of hexoses and pentoses is of importance for their uptake in muscle. Insulin is able to increase the uptake of D-glucose, D-fructose, D-galactose, L-arabinose, and some other sugars, whereas the uptake of D-arabinose, for example, is uninfluenced by insulin (PARK *et al.*, 1955, 1957; HAFT, MIRSKY and PERISUTTI, 1953). The results with isolated rat spinal cord indicate that the same distinction between insulin responsive and insulin non-responsive sugars is valid in this preparation, as insulin increased the uptake of D-glucose, D-fructose, and D-galactose, but had no effect on the uptake of D-arabinose.

Glucose uptake determinations have not been much used in experiments with isolated samples from the central nervous system and all in all it is difficult to find results in the literature with which the present ones are directly comparable. WINTERSTEIN and HIRSCHBERG (1925) incubated isolated frog central nervous system and found that insulin *in vitro* increased the content of glycogen and cerebrosides in this preparation. An increase in glycogen of more than 100 per cent and in cerebrosides of more than 200 per cent was seen when insulin in 'moderate' concentrations was added to the medium, whereas insulin in high concentrations caused a decrease of both glycogen and cerebrosides. On the other hand, glycogen synthesis in guinea-pig brain slices was not stimulated by addition of insulin to the incubation medium (LEBARON, 1955; MCILWAIN and TRESIZE, 1956). The glucose uptake of rat brain slices was studied by BELOFF-CHAIN *et al.* (1956) in isotope experiments. Glucose uptake calculated from the decrease of total counts in the medium during incubation or from activity of all fractions analysed in the brain slice plus activity in carbon dioxide produced was uninfluenced by the presence of insulin in the incubation medium. In the same experiments no effect of insulin was found on the conversion of labelled glucose in the medium to labelled carbon dioxide. WOODS, HUNTER and BURK (1958) measured the anaerobic glycolysis of mouse brain slices, and they found that aerobic pre-incubation with insulin increased the subsequent anaerobic glycolysis by up to 30 per cent in half of the experiments.

It was also found that in experiments where insulin was injected intraperitoneally in rats an effect on the glucose uptake of isolated spinal cord pieces was seen during the subsequent incubation, even after an interval of only 15 minutes from injection to isolation of the spinal cord. The increased glucose uptake might be due to a reduction of some of the intracellular metabolites in the spinal cord caused by an unchanged consumption in the spinal cord at a time when glucose supply *via* the blood was decreased by the injection of insulin. This is unlikely since the spinal cord pieces also showed a considerably increased glucose uptake in experiments where the blood glucose values of the rats at death were only slightly reduced. The increase of glucose uptake is more likely to be due to insulin which has rapidly reached the central nervous system. The evidence that ^{131}I-labelled insulin does not enter the central nervous system is not convincing (SCOTT *et al.*, 1958; BRUNFELDT, 1960).

In this study and the following ones (RAFAELSEN, 1961*a*, RAFAELSEN and CLAUSEN, 1961) insulin has been shown to increase the glucose uptake *in vitro* of some isolated samples from the central nervous system of the rat: rat spinal cord pieces, 'first' brain slices, and isolated cerebellar tissue in that order of efficacy. The effect resembles in many ways the effect of insulin on other isolated samples such as rat diaphragm and rat epididymal fat. It is evident that the central nervous system normally receives its nutrients *via* the vascular bed and not from the cerebrospinal

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fluid bathing its surfaces. The problem of the supply of nutrients is, however, of equal importance in experiments with other isolated tissues.

SUMMARY

1. The uptake of carbohydrates by isolated rat spinal cord has been studied. The glucose uptake was dependent on the glucose concentration in the medium, and the uptake was not altered by homogenization of the spinal cord.

2. Insulin increased the glucose uptake of isolated spinal cord pieces incubated aerobically by 40 per cent when present in the medium in a concentration of 10^{-4} i.u./ml, and by 80 per cent when present in a concentration of 10^{-1} i.u./ml.

3. Glucose uptake of spinal cord pieces incubated in media without insulin was increased when the rats were given an injection of insulin from 15 to 120 minutes before sacrifice.

4. Insulin increased the uptake of D-fructose and D-galactose, but was without an effect on the uptake of D-arabinose.

5. Insulin had no effect on the glucose uptake of isolated spinal cord pieces during anaerobic incubation, nor did it affect the glucose uptake of rat spinal cord homogenate.

6. The hypothesis is advanced that the relative intactness of the tissue samples and the preservation of intact organ membranes over the larger part of the surface is of great importance for the demonstration of a direct effect of insulin on the carbohydrate uptake of the central nervous system.

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ACTION OF INSULIN ON GLUCOSE UPTAKE OF RAT BRAIN SLICES AND ISOLATED RAT CEREBELLUM

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RAT spinal cord can be isolated for incubation experiments, and insulin added *in vitro* or injected *in vivo* increases the glucose uptake of isolated rat spinal cord during incubation (RAFAELSEN, 1958, 1961). The spinal cord preparation was chosen as a sample of the central nervous system which could be isolated relatively intact and which, during incubation, would still be covered with natural organ membranes over the greater part of the surface. It was supposed that a direct effect of insulin *in vitro* was most easy to demonstrate on tissue samples which were as intact as possible and it was thought that the preservation of natural organ membranes was especially important. From these aspects the spinal cord was well suited for incubation studies, and after the demonstration of a direct effect of insulin on the uptake of glucose and some other sugars in the isolated rat spinal cord it was natural to try to demonstrate a direct effect of insulin on the glucose uptake of other isolated samples of the central nervous system. This paper consists of a description of the findings using rat brain slices and isolated rat cerebellar tissue.

EXPERIMENTAL

Animals. Female, non-pregnant, albino rats (Wistar strain), weighing 150–350 g and fasted for 24 hr were used.

Anaesthesia. Anaesthesia was induced by inhalation of a mixture of 50% CO_2 and 50% O_2 for 2–3 min.

Procedure. The anaesthetized animals were decapitated with a strong pair of scissors, the skull carefully divided in the midline and the brain removed *in toto*. In the brain slice experiments the hemispheres of the brain were immediately sliced with a Stadie-Riggs slicer (STADIE and RIGGS, 1944, modified with a quick-release gear). One or two slices were cut, always from the convexity of each hemisphere. The first brain slice cut from a hemisphere has an intact, pia-covered surface on one side and a cut surface on the other side, whereas the second brain slice has two cut surfaces. In the following they will be referred to as 'first' and 'second' slices. The slices were at once placed in the incubation flasks. Each brain slice was incubated separately, wet weights immediately after slicing varying from 60 to 125 mg, thickness about 0·3 mm. In the cerebellum experiments the brachia cerebelli were cut as near the cerebellum as possible, using a thin-bladed scalpel. The cerebellum was incubated separately *in toto*, wet weights immediately after isolation varying from 200 to 250 mg.

The incubation medium, incubation procedure, glucose determination, and statistical analysis were exactly as reported for the rat spinal cord experiments (RAFAELSEN, 1958, 1961).

After incubation two samples of 0·100 ml were withdrawn from the incubation medium in each flask for glucose determination. Samples were also withdrawn from control flasks aerated and incubated without tissue. Glucose uptake was calculated from the difference between the glucose concentration in the control flasks and that in the flasks containing brain tissue. The uptake was expressed as mg of glucose/g of wet brain slice or cerebellum/hr.

In each experiment 8–12 control brain slices or isolated cerebella were incubated simultaneously with 8–12 brain slices or isolated cerebella under the influence of insulin. Mean values for glucose uptake were calculated for control tissues and for tissues under the influence of insulin.

In the brain slice experiments the rats used were always untreated. All the brain slices incubated in a single experiment were either first brain slices or second brain slices. 'Pairing' of brain slices was performed so that from each animal one slice was incubated in medium with insulin and that from the other hemisphere of the same rat in medium without insulin. Glucagon-free insulin containing 27 international units (i.u.) per mg was employed in concentrations from 10^{-4} to 10^{-1} i.u./ml.

With cerebellar tissue two main types of experiments were performed. In one type (parallel to the brain slice experiments), cerebellar tissue from untreated rats were incubated in medium with or without insulin. Insulin was employed in concentrations of from 10^{-3} to 1 i.u./ml. In the other type of cerebellum experiments, cerebellar tissue from rats given an intraperitoneal injection of either insulin or saline before sacrifice were incubated in medium without insulin. The volume injected was always 1 ml, and insulin was given in a dose of 1 i.u./100 g body weight 60 min before sacrifice.

Dry weight. Wet weights before and after incubation were determined in some of the brain slice experiments. Dry weights were determined in the same experiments after desiccation of the tissue pieces over phosphorus pentoxide for 48 hr.

RESULTS

Glucose uptake

Glucose uptake of second brain slices was somewhat higher than that of first brain slices (Table 1). The mean glucose uptake of 59 control second brain slices

TABLE 1.—GLUCOSE UPTAKE OF BRAIN SLICES FROM UNTREATED RATS. FIRST SLICES VERSUS SECOND SLICES

Expt. no.	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet brain slice/hr) First slices	Second slices	Difference (mg of glucose/g of wet brain slice/hr)	(%)	Significance of difference (*P*)
160	8·9 ± 0·25 (12)	7·8 ± 0·23 (12)	−1·1	−12	*<0·01*
166	7·4 ± 0·31 (10)	9·5 ± 0·42 (10)	+2·1	+28	*<0·001*
171	8·3 ± 0·33 (10)	10·0 ± 0·34 (10)	+1·7	+21	*<0·01*
179	8·0 ± 0·22 (11)	11·4 ± 0·31 (11)	+3·4	+43	*<0·001*
180	10·7 ± 0·24 (11)	11·3 ± 0·19 (11)	+0·6	+6	>0·05

The number of brain slices is given in parentheses.

incubated in medium with 300 mg of glucose/100 ml was 9·96 ± 0·22 mg of glucose/g of wet brain slice/hr, and that of 54 control first brain slices incubated in the same medium was 8·69 ± 0·19 mg of glucose/g of wet brain slice/hr. The difference between the mean glucose uptake was 1·27 ± 0·29 mg of glucose/g of wet brain slice/hr ($P < 0·001$).

First and second brain slices differed also in their ability to increase their glucose uptake when insulin was present in the medium. Table 2 shows the effect of insulin on glucose uptake of first brain slices from untreated rats. It is seen that insulin in a concentration of 10^{-1} i.u./ml in the medium increased the glucose uptake by 10 to 15 per cent. The increase was statistically significant. With one exception insulin in lower concentrations was unable to increase the glucose uptake of first brain slices significantly. Table 3 shows that insulin even in a concentration of 10^{-1} i.u./ml in the medium was unable to increase the glucose uptake significantly in the case of second brain slices.

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TABLE 2.—GLUCOSE UPTAKE OF FIRST BRAIN SLICES FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM

Expt. no.	Conc. of insulin in medium (i.u./ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet brain slice/hr) Without insulin	With insulin	Difference (mg of glucose/g of wet brain slice/hr)	(%)	Significance of difference (*P*)
135	10^{-1}	7·3 ± 0·30 (11)	8·4 ± 0·36 (11)	+1·1	+15	*<0·05*
166	10^{-1}	7·4 ± 0·31 (10)	8·5 ± 0·29 (10)	+1·1	+15	*<0·02*
179	10^{-1}	8·0 ± 0·22 (11)	9·3 ± 0·24 (11)	+1·3	+16	*<0·001*
180	10^{-1}	10·7 ± 0·24 (11)	11·6 ± 0·26 (11)	+0·9	+8	*<0·02*
160	10^{-2}	8·9 ± 0·25 (12)	9·7 ± 0·28 (12)	+0·8	+9	*<0·05*
168	10^{-2}	10·0 ± 0·30 (10)	10·3 ± 0·33 (10)	+0·3	+3	>0·05
171	10^{-2}	8·3 ± 0·33 (10)	8·4 ± 0·33 (10)	+0·1	+1	>0·05
189	10^{-2}	8·9 ± 0·22 (12)	8·9 ± 0·30 (12)	0	0	—
190	10^{-2}	12·5 ± 0·29 (12)	12·2 ± 0·27 (12)	−0·3	−2	>0·05
139	10^{-4}	6·9 ± 0·29 (12)	7·2 ± 0·29 (12)	+0·3	+4	>0·05

The number of brain slices is given in parentheses.

TABLE 3.—GLUCOSE UPTAKE OF SECOND BRAIN SLICES FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM

Expt. no.	Conc. of insulin in medium (i.u./ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet brain slice/hr) Without insulin	With insulin	Difference (mg of glucose/g of wet brain slice/hr)	(%)	Significance of difference (*P*)
166	10^{-1}	9·5 ± 0·42 (10)	9·9 ± 0·15 (10)	+0·4	+4	>0·05
179	10^{-1}	11·4 ± 0·31 (10)	10·9 ± 0·30 (11)	−0·5	−4	>0·05
180	10^{-1}	11·3 ± 0·19 (11)	11·8 ± 0·40 (11)	+0·5	+4	>0·05
160	10^{-2}	7·8 ± 0·23 (12)	8·0 ± 0·37 (12)	+0·2	+3	>0·05
171	10^{-2}	10·0 ± 0·34 (10)	10·1 ± 0·25 (10)	+0·1	+1	>0·05

The number of brain slices is given in parentheses.

Cerebellar tissue incubated *in toto* had a definite glucose uptake in the absence of insulin. The mean glucose uptake of 58 control cerebella incubated in a medium with 300 mg of glucose/100 ml was 3·17 ± 0·05 mg of glucose/g of wet cerebellum/hr.

Table 4 shows the effect of insulin added to the incubation medium on the glucose uptake of isolated cerebellar tissue. It is seen that insulin in high concentrations increased the glucose uptake by about 10 per cent. The increase was statistically significant. In the second type of experiment, 1 i.u. of insulin was injected intraperitoneally into the rats 60 minutes before sacrifice. The glucose uptake of cerebellar tissue from these animals was compared with the glucose uptake of cerebellar tissue from control animals injected with saline. In these experiments no insulin was added to the medium. It is seen that the injection of insulin caused a small, but statistically significant increase of the glucose uptake in these experiments (Table 5).

TABLE 4.—GLUCOSE UPTAKE OF CEREBELLUM FROM UNTREATED RATS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM

Expt. no.	Conc. of insulin in medium (i.u./ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet cerebellum/hr) Without insulin	With insulin	Difference (mg of glucose/g of wet cerebellum/hr)	(%)	Significance of difference (*P*)
212	10^{0}	3·3 ± 0·07 (12)	3·6 ± 0·05 (12)	+0·3	+9	*~0·001*
210	10^{-1}	2·8 ± 0·04 (11)	3·0 ± 0·04 (12)	+0·2	+7	*<0·001*
213	10^{-1}	2·9 ± 0·06 (12)	3·2 ± 0·05 (12)	+0·3	+10	*<0·01*
214	10^{-2}	3·0 ± 0·16 (11)	3·0 ± 0·13 (12)	0	0	—
215	10^{-3}	3·7 ± 0·07 (12)	3·7 ± 0·07 (12)	0	0	—

The number of cerebella is given in parentheses.

TABLE 5.—GLUCOSE UPTAKE OF CEREBELLUM FROM CONTROL RATS TREATED WITH SALINE AND RATS TREATED WITH INSULIN. 1 i.u. INSULIN/100 g BODY WEIGHT INJECTED INTRAPERITONEALLY 60 MINUTES BEFORE SACRIFICE

Expt. no.	Blood sugar at sacrifice Mean and range (mg/100 ml)	Glucose uptake Mean ± S.E.M. (mg of glucose/g of wet cerebellum/hr) Saline treated animals	Insulin treated animals	Difference (mg of glucose/g of wet cerebellum/hr)	(%)	Significance of difference (*P*)
216	14 (10–18)	3·1 ± 0·05 (12)	3·3 ± 0·06 (12)	+0·2	+6	*~0·02*
217	11 (8–21)	2·4 ± 0·06 (12)	2·6 ± 0·07 (12)	+0·2	+8	*~0·02*
219	17 (9–26)	3·4 ± 0·07 (12)	3·6 ± 0·08 (12)	+0·2	+6	*~0·05*

The number of cerebella is given in parentheses.

Solid matter content

A percentage dry weight of 23 (n : 32) was found in brain slices immediately after isolation. After soaking and incubation the percentage dry weight had fallen to 14 (n : 32). With no loss of tissue fragments an increase in wet weight during soaking and incubation of some 60 per cent was foreseable from the changes in percentage dry weight observed. Actually, an increase in wet weight of only 25 per cent was observed (n : 32). The brain slices thus suffer a considerable loss in the form of debris or tissue fragments during incubation. First and second brain slices had the same original percentage dry weight and the swelling during incubation was also of the same order of magnitude.

DISCUSSION

In earlier studies it was found that insulin increased the glucose uptake of isolated rat spinal cord (RAFAELSEN, 1958, 1961). It was natural to see whether insulin also had a direct effect on the glucose uptake of other isolated samples from the central nervous system. This subject has been but little investigated. Brain slices were chosen because a large amount of knowledge has accumulated from the widespread use of this sample.

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Nevertheless, brain slices are a much less uniform material for metabolic studies than are slices from a homogenous organ, e.g. the liver. First brain slices have, apart from the cytoarchitecture, the special feature that one side is intact, covered with meninges, whereas following brain slices have two artificial, cut surfaces. First and second brain slices vary in a number of ways both with regard to initial content of different constituents and to activity during incubation. The glycogen content is unevenly distributed in the brain hemisphere of guinea pigs. The superficial parts of the hemisphere is appreciably richer than the inner parts, the values falling from first to second to third slices (LEBARON, 1955; MCILWAIN and TRESIZE, 1956). The lipid content of rat brain is some 35 per cent higher in second slices than in first slices (MAJNO and KARNOVSKY, 1958). Also during incubation the different brain slices exhibit different properties. As shown in this paper, the glucose uptake of second brain slices is somewhat higher than that of first brain slices. MAJNO and KARNOVSKY (1958) found that the incorporation of ^{14}C-labelled acetate into lipids is twice as high in first brain slices as in second slices, whereas the oxygen uptake is not significantly different. Glycogen present in brain slices disintegrates rapidly after excision of tissue samples from the brain, but brain slices can resynthetize glycogen during incubation and here also the different brain slices have quantitatively different properties. The resynthesis of glycogen during incubation of guinea pig brain slices is highest in first slices and lowest in third slices, second slices occupying an intermediate position, according to LEBARON (1955) and MCILWAIN and TRESIZE (1956).

Very often brain slices disintegrate to some extent during incubation and it is known that the slices swell, i.e. take up fluid from the incubation medium, to a considerable degree. From the present observations of alterations of percentage dry weight between isolation and the end of incubation, and from the weight of the brain slices, it was calculated that the brain slices lost 20 per cent of their original substance during soaking and incubation and that the swelling amounted to 60 per cent (it should be noted that the net increase in wet weight was only 25 per cent). In comparison it was found that rat spinal cord pieces only swelled 15 per cent and had a mean loss of substance of 5 per cent. ELLIOTT (1946), working with brain slices, found that soaking alone for 20 minutes led to a mean loss of substance of 8 per cent; PAPPIUS and ELLIOTT (1956) found that rat brain slices swelled 40 per cent during one hour's incubation in glucose-containing medium.

It is apparent that brain slices have a good surface—weight ratio and a thickness of about 0·3 to 0·4 mm which allows full oxygenation when the tissue sample is incubated in an atmosphere of oxygen. On the other hand, the apparent surface : weight ratio is unfavourable in the cerebellum, but it should be remembered that in man and many animals the real surface is about 6 times as great as the area of the cerebellar convexities, due to the folia cerebelli. From a theoretical point of view, complex diffusion problems are encountered in connection with incubation of isolated rat cerebellum, as this sample has a diameter of about 5 mm. The problems are parallel to those encountered with incubation of sympathetic rat ganglia (LARRABEE, 1958) and isolated rat spinal cord (RAFAELSEN, 1961). These preparations have been very useful in spite of the probable existence of an 'anoxic' or 'glucose-poor' core in the interior of the preparations.

The glucose uptake of first brain slices was 10 to 15 per cent lower than that of second brain slices. This observation, together with observations by other workers of

different properties of first and second brain slices, stresses the importance of using either first brain slices or second brain slices for incubation experiments. The glucose uptake of isolated cerebellar tissue was only one third of that of brain slices and this is perhaps due to the much more unfavourable surface : weight ratio, but functional differences may also play a role. The glucose uptake of isolated cerebellum was in the same range as in isolated rat spinal cord pieces (RAFAELSEN, 1961).

Insulin *in vitro* caused a moderate increase in the glucose uptake of first brain slices but no increase in that of second brain slices. The effect of insulin on glucose uptake of brain slices seems scarcely to have been investigated with classical chemical methods by other workers. Using an isotope technique, BELOFF-CHAIN *et al.* (1956) found no effect of 1 i.u. insulin/ml on the disappearance of labelled glucose from the incubation medium. These workers incubated first and second brain slices together in the same flask. This will, of course, reduce the possibility of demonstrating a moderate effect of insulin taking place only in first brain slices. Furthermore, the glucose uptake was calculated from the observations made in a manner which may have obscured the results. The glucose uptake was calculated from (1) the decrease of counts in the medium during incubation, and (2) from the activity of all fractions analysed in the brain slice plus the activity in the carbon dioxide produced. The complexity of the second procedure and the great scatter of the percentage distribution makes it difficult to observe a small increase of glucose uptake due to insulin. In the first procedure, it is evident that a more pronounced decrease of isotopically labelled glucose in the medium due to insulin will only be found if isotopically labelled metabolites do not appear more abundantly in the medium at the same time. This possibility was not excluded in the experiments by BELOFF-CHAIN and coworkers. These workers were also unable to demonstrate changes due to insulin in the amounts of the various fractions analysed in the brain slices or in the labelled carbon dioxide produced. WOODS, HUNTER and BURK (1958) observed that aerobic preincubation of brain slices with $1{\cdot}5 \times 10^{-1}$ i.u. insulin/ml increased the subsequent anaerobic glycolysis in modified Krebs–Ringer solution by up to 30 per cent in half of the experiments performed. It was not reported whether first and second brain slices were used sorted or unsorted. Insulin is not able to stimulate the glycogen synthesis of isolated samples from the central nervous system. LEBARON (1955) found no effect of insulin *in vitro* on glycogen synthesis in guinea pig brain slices, and the results of MCILWAIN and TRESIZE (1956) were also negative in five out of six experiments. In one experiment, these workers saw a small, but statistically significant increase of glycogen synthesis in the presence of insulin.

The effect of insulin on the glucose uptake of isolated cerebellum is evidently a direct one when the hormone is added to the incubation medium *in vitro*. When insulin is injected into the rat and the cerebellum isolated some time later and incubated in a medium without insulin, it is more difficult to decide whether the increased glucose uptake is due to a direct or an indirect effect of insulin on the central nervous system. The completely parallel problem in connection with incubation of spinal cord pieces isolated from insulin-treated rats has been discussed in the preceding paper (RAFAELSEN, 1961).

In this and foregoing papers it has been shown that insulin *in vitro* increases the glucose uptake of isolated spinal cord pieces, first brain slices, and isolated cerebellar

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tissue from rats, whereas this hormone has no effect on the glucose uptake of spinal cord homogenate or second brain slices. It is suggested that the positive results obtained are connected to the presence of natural organ membranes over a larger part of the surface of these tissue samples. The effect of insulin *in vitro* on the glucose uptake of structurally intact samples from other organ systems lends support to this view.

SUMMARY

1. Glucose uptake of 'second' brain slices was 10 to 15 per cent greater than that of 'first' brain slices from rats.

2. Insulin (10^{-1} i.u./ml) increased the glucose uptake of first brain slices by 10 to 15 per cent, whereas it had no effect on the glucose uptake of second brain slices.

3. Insulin increased the glucose uptake of isolated rat cerebellum when added *in vitro*.

4. Cerebellar tissue isolated from rats which one hour before sacrifice had been injected with insulin also showed an increased glucose uptake during subsequent incubation.

5. It is suggested that the direct effect of insulin on glucose uptake of first brain slices and isolated cerebellar tissue and—as reported earlier—of isolated rat spinal cord is connected to the preservation of intact organ membranes over a large part of the surface of these samples during incubation.

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FATE OF GLUCOSE IN ISOLATED RAT SPINAL CORD AND DIAPHRAGM INCUBATED IN THE ABSENCE AND PRESENCE OF INSULIN

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IT HAS been shown in previous reports that insulin *in vitro* increased the glucose uptake of isolated spinal cord pieces, 'first' brain slices, and isolated cerebellar tissue from rats (RAFAELSEN, 1958, 1961*a*, 1961*b*). In spinal cord the increased glucose uptake due to insulin was 40 to 80 per cent, but was only 10 to 15 per cent in experiments with first brain slices and isolated cerebellum.

This report is of a study of the fate of the extra glucose taken up by isolated rat spinal cord in the presence of insulin. It was investigated whether or not insulin influenced the conversion of glucose from the medium to carbon dioxide and increased the lactate accumulation in the medium. The questions as to whether or not the glycogen content in spinal cord pieces after incubation was greater when insulin had been present in the incubation medium, and whether or not the extra glucose taken up was incorporated into the glycolipids or cerebrosides were also investigated.

For the sake of comparison parallel experiments with isolated rat diaphragms were included in the study, except that the incorporation of radioactive glucose into glycolipids was not investigated with the muscle sample.

EXPERIMENTAL

Animals. Female, non-pregnant, albino rats (Wistar strain) weighing 80–100 g and fasted for 24 hr were used.

Anaesthesia. Anaesthesia was induced by inhalation of a mixture of 50% CO_2 and 50% O_2 for 2–3 min.

Procedure. The spinal cords or hemidiaphragms were carefully isolated and incubated for 90 mins exactly as described earlier (RAFAELSEN, 1958, 1959, 1961*a*). Glucose was added to the medium of GEY and GEY (1936) to a final concentration of 300 mg/100 ml. In some of the experiments isotopically labelled glucose was also added to the incubation medium. Glucagon-free insulin containing 27 international units (i.u.) per mg was employed in a concentration of 10^{-1} i.u./ml.

In each experiment 8–12 spinal cord pieces or hemidiaphragms were incubated simultaneously with 8–12 spinal cord pieces or hemidiaphragms under the influence of insulin. Pieces of similar size were 'paired' for incubation in flasks without and with insulin, but no attempt was made to pair spinal cord pieces belonging to the same animal. Mean values of glycogen content, lactic acid production, or carbon dioxide production were calculated for control spinal cord pieces or hemidiaphragms and for spinal cord pieces or hemidiaphragms under the influence of insulin. All values in the Tables are given $\pm$ S.E.M. (standard error of the estimated mean). The significance of the difference between means has been established by calculating 't'. *P*, the probability of difference being due to chance, was obtained from tables for 't' (FISHER and YATES, 1943). In the experiments where incorporation of radioactivity into lipids was measured, the determinations themselves were mean values as the spinal cord pieces in the experiments had been pooled.

Four types of experiment were performed:

(1) *Glycogen content.* Determination of glycogen content in the tissue samples after incubation. In these experiments each tissue sample immediately after the incubation was dissolved in 0·5 ml of boiling 30% KOH and the glycogen precipitated by adding 1 ml 96% ethanol as described by LEBARON (1955). After hydrolysis for 2·5 hr with N-HCl glucose was determined by the glucose oxidase method (KEILIN and HARTREE, 1948). Glycogen content was expressed as mg of glycogen/g of wet spinal cord or diaphragm.

(2) *Lactic acid production in medium.* After incubation a sample of 1·000 ml was withdrawn from the incubation medium in each flask for duplicate lactic acid determinations. Samples were also withdrawn from control flasks aerated and incubated without tissue. These samples served as blanks. Lactic acid was determined by the lactic acid dehydrogenase method of HORN and BRUNS (1956). Lactic acid production was expressed as mg of lactic acid/g of wet spinal cord or diaphragm.

(3) CO_2 *production from glucose in the medium during incubation.* In these experiments labelled D-glucose was added to the incubation medium to a final concentration of 0·05 μc/ml. [U-^{14}C] glucose and [1-^{14}C] glucose were delivered from the Radiochemical Centre, Amersham, England. The specific activity of the batches was about 10 mc/m-mole. The incubation flasks were always closed with a rubber stopper with two holes in which were placed glass tubes. The outer ends of the glass tubes were connected to rubber tubing and during incubation a pinchcock isolated the atmosphere in the flask from the surroundings. A stainless steel planchet with a diameter of 13 mm was placed in a holder 1 cm below the rubber stopper inside the flask. After incubation the flasks were placed in an icebath and 0·15 ml N-NaOH were injected via one of the tubes onto the steel planchet, and 0·2 ml 10 N-H_2SO_4 via the other tube into the incubation medium. The CO_2 liberated from the medium was absorbed in the NaOH during the next two hours (at room temperature). After this the steel planchets were carefully removed from the holders and dried for 12 hr at 37°, for 2 hr at 60°, and finally at 160° for 1 hr.

The carbonate samples were counted in a gas-flow proportional counter (Frieseke and Hoepfner type 90 + 449 + 516). In each experiment two flasks without tissue served as blanks. The activity was expressed as counts per minute/g of wet spinal cord or diaphragm.

(4) *Incorporation of radioactivity from* [U-^{14}C] *glucose into lipids of spinal cord tissue.* In these experiments [U-^{14}C] glucose was added to the medium to a final concentration of 0·16 μc/ml. After incubation the spinal cord pieces were washed rapidly in three changes of medium in order to remove radioactive glucose from the exterior of the pieces. Then all spinal cord pieces incubated without insulin were pooled as were all pieces incubated with insulin. The lipids were extracted by homogenizing the tissue in a POTTER-ELVEHJEM homogenizer with 50 vol. chloroform–methanol 2:1 (v/v). The crude extract, after filtration, was thoroughly mixed with 10 vol. 0·1% saline, and allowed to separate by standing for 72 hr or by centrifuging for 15 min at 4000 rev/min as described by FOLCH, LEES and SLOANE STANLEY (1957). A biphasic system was obtained. Samples from the upper and lower phase were plated on planchets with a diameter of 18 mm for radioactive measurements. In addition, 10 ml samples were dialysed against running tap water for 24 to 48 hr and then plated for radioactivity measurements. All planchets were dried at 37° for 12 hr, then at 60° for 2 hr, and at 110° for 2 hr.

The counting was as described in the experiments on CO_2. The activity was expressed as counts per minute/g of wet spinal cord.

RESULTS

Glycogen content

The glycogen content of rat spinal cord pieces after incubation was very low: 0·2 to 0·4 mg of glycogen/g of wet spinal cord (Table 1). Insulin in a concentration of 10^{-1} i.u./ml in the medium had no effect on the amount of glycogen present after incubation.

In diaphragms isolated from rats fasted and treated in exactly the same way as the rats used for the spinal cord experiments, the mean glycogen content after incubation in glucose-containing medium was 3·0 mg of glycogen/g of wet diaphragm. Addition of insulin to the medium to a final concentration of 10^{-4} i.u./ml did not

TABLE 1.—GLYCOGEN CONTENT OF SPINAL CORD PIECES AND HEMIDIAPHRAGMS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM (FROM UNTREATED RATS)

Expt. no.	Insulin conc. in medium (i.u./ml)	Glycogen content Mean ± S.E.M. (mg of glycogen/g of wet tissue) Without insulin	With insulin	Difference (mg of glycogen/g of wet tissue)	(%)	Significance of difference (*P*)
SPINAL CORD						
244	10^{-1}	0·40 ± 0·03 (8)	0·38 ± 0·02 (7)	−0·02	−5	>0·05
245	10^{-1}	0·22 ± 0·01 (11)	0·23 ± 0·01 (11)	+0·01	+5	>0·05
247	10^{-1}	0·23 ± 0·01 (11)	0·25 ± 0·01 (12)	+0·02	+9	>0·05
252	10^{-1}	0·23 ± 0·01 (12)	0·24 ± 0·01 (12)	+0·01	+4	>0·05
293	10^{-2}	0·42 ± 0·03 (10)	0·44 ± 0·02 (11)	+0·02	+5	>0·05
DIAPHRAGM						
51	10^{-4}	4·3 ± 0·1 (6)	4·0 ± 0·3 (6)	−0·3	−7	>0·05
89	10^{-4}	1·9 ± 0·1 (8)	2·0 ± 0·3 (8)	+0·1	+5	>0·05
91	10^{-4}	1·5 ± 0·2 (8)	1·7 ± 0·2 (8)	+0·2	+13	>0·05
95	10^{-4}	1·4 ± 0·1 (8)	1·8 ± 0·2 (8)	+0·4	+29	>0·05
56	10^{-2}	3·6 ± 0·3 (6)	5·8 ± 0·4 (6)	+2·2	+61	*<0·01*
89	10^{-2}	1·9 ± 0·1 (8)	2·7 ± 0·4 (8)	+0·8	+42	*<0·05*
91	10^{-2}	1·5 ± 0·2 (8)	2·6 ± 0·4 (8)	+1·1	+73	*<0·05*
95	10^{-2}	1·4 ± 0·1 (8)	2·5 ± 0·5 (8)	+1·1	+79	*~0·05*

The number of spinal cord pieces or hemidiaphragms is given in parentheses.

significantly increase the glycogen content present after incubation, but insulin 10^{-2} i.u./ml increased it by 60 per cent (Table 1).

Lactic acid production in medium

After incubation of isolated rat spinal cord pieces for 90 min, the medium contained about 1·5 mg of lactic acid/g of wet spinal cord. The glucose uptake of the spinal cord pieces in parallel experiments was 4–5 mg of glucose/g of wet spinal cord (~3·0 to 3·5 mg of glucose/g of wet spinal cord/hr). About one third of the glucose taken up had thus been liberated into the medium as lactic acid. The presence of insulin in the medium had no effect on the amount of lactic acid liberated into the medium (Table 2), nor did insulin exert an action on the liberation of lactic acid by rat diaphragm under the experimental conditions used.

Conversion of [U-^{14}C]*glucose and* [1-^{14}C]*glucose to* [^{14}C]*carbon dioxide*

No effect of insulin on the conversion of isotopically labelled glucose to carbon dioxide by spinal cord pieces was observed, either with [U-^{14}C]glucose or with [1-^{14}C]glucose added to the medium (Table 3). Insulin was also without effect on the conversion of [U-^{14}C]glucose to [^{14}C]carbon dioxide in isolated rat diaphragm (Table 3).

Incorporation of ^{14}C *from* [U-^{14}C]*glucose in lipid fractions*

When the extraction procedure of FOLCH, LEES and SLOANE STANLEY (1957) is used an upper phase of methanol–water (1 : 1) is obtained. This constitutes some 40 per cent of the volume. The lower phase constitutes some 60 per cent and is a

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TABLE 2.—LACTIC ACID CONTENT OF MEDIUM AFTER INCUBATION OF SPINAL CORD PIECES OR HEMIDIAPHRAGMS FROM UNTREATED RATS WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM. INSULIN CONCENTRATION IN MEDIUM: 10^{-1} i.u./ml.

Expt. no.	Lactic acid content Mean ± S.E.M. (mg of lactic acid in medium/g of wet tissue) Without insulin	With insulin	Difference (mg of lactic acid in medium/g of wet tissue)	(%)	Significance of difference (P)
SPINAL CORD					
292	1·9 ± 0·10 (9)	2·0 ± 0·12 (9)	+0·1	+5	>0·05
286	1·6 ± 0·15 (10)	1·5 ± 0·14 (10)	−0·1	−6	>0·05
287	1·5 ± 0·17 (10)	1·7 ± 0·17 (10)	+0·2	+13	>0·05
289	1·7 ± 0·25 (9)	1·6 ± 0·28 (9)	−0·1	−6	>0·05
291	1·8 ± 0·18 (9)	1·7 ± 0·15 (9)	−0·1·	−6	>0·05
DIAPHRAGM					
294	4·5 ± 0·38 (8)	5·1 ± 0·41 (8)	+0·6	+13	>0·05
295	3·6 ± 0·23 (8)	4·1 ± 0·30 (8)	+0·5	+14	>0·05
298	3·6 ± 0·59 (8)	3·8 ± 0·52 (8)	+0·2	+6	>0·05

The number of spinal cord pieces or hemidiaphragms is given in parentheses.

TABLE 3.—CONVERSION OF [U-^{14}C] GLUCOSE AND [1-^{14}C] GLUCOSE TO $^{14}CO_2$ BY SPINAL CORD PIECES AND HEMIDIAPHRAGMS INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM (FROM UNTREATED RATS)
INSULIN CONCENTRATION IN MEDIUM: 10^{-1} i.u./ml.

Expt. no.	Carbon dioxide Mean ± S.E.M. (counts/min/g of wet tissue) Without insulin	With insulin	Difference (counts/min/g of wet tissue)	(%)	Significance of difference (P)	
SPINAL CORD						
302	3620 ± 202 (8)	3425 ± 148 (8)	−195	−5	>0·05	[U-^{14}C] labelled D-glucose
303	3562 ± 146 (11)	3390 ± 143 (11)	−172	−5	>0·05	
304	2130 ± 121 (9)	1873 ± 170 (10)	−257	−12	>0·05	
307	2055 ± 214 (10)	2131 ± 214 (10)	+76	+4	>0·05	[1-^{14}C] labelled D-glucose
308	1837 ± 113 (10)	1973 ± 158 (10)	+136	+7	>0·05	
DIAPHRAGM						
305	1652 ± 91 (9)	1666 ± 87 (9)	+14	+1	>0·05	[U-^{14}C]labelled D-glucose
306	1635 ± 97 (10)	1688 ± 85 (8)	+53	+3	>0·05	

The number of spinal cord pieces or hemidiaphragms is given in parentheses.

13 : 3 mixture of chloroform and methanol. It is seen from Table 4 that most of the radioactivity was found in the upper phase. In this phase by far the greater part of the radioactivity was present in low molecular metabolites, as only about 1/50 was left after dialysis. It is seen that the total activity of the dialysed upper phase tended to increase in spinal cord pieces incubated in the presence of insulin, but the increase in the four experiments was not statistically significant. On the other hand, the activity left after dialysis was some 40 per cent lower in spinal cord pieces which had been under the influence of insulin *in vitro*. This decrease was statistically significant ($P < 0·02$).

TABLE 4.—INCORPORATION OF [^{14}C] FROM [U-^{14}C] GLUCOSE INTO LIPID FRACTIONS BY SPINAL CORD PIECES INCUBATED WITHOUT AND WITH INSULIN ADDED TO THE MEDIUM FROM UNTREATED RATS
INSULIN CONCENTRATION IN MEDIUM: 10^{-1} i.u./ml.

Expt. no.	Radioactivity (counts/min/g of wet spinal cord) Without insulin	With insulin	Difference (counts/min/g of wet spinal cord)	(%)	Significance of difference (*P*)
	Upper phase undialysed				
288	48550	49350	+800	+2	
290	47333	52050	+4717	+9	
296	36800	47725	+10925	+23	
298	39450	44916	+5466	+12	
	43033	48510	+5477	+11	>0·05(<0·10)
	Upper phase dialysed				
288	141	85	−56	−40	
290	508	201	−307	−60	
296	47	35	−12	−26	
298	153	102	−51	−33	
	212	106	−106	−42	*<0·02*
	Lower phase undialysed				
288	2165	2500	+335	+16	
290	4150	3680	−470	−11	
296	3333	2318	−1015	−30	
298	9450	6133	−3317	−35	
	4774	3657	−1117	−23	>0·05
	Lower phase dialysed				
290	2235	2485	+250	+11	
296	1235	1580	+345	+28	
298	2370	1435	−935	−39	
	1946	1833	−113	−6	>0·05

The total activity of the undialysed lower phase tended to be decreased in spinal cord pieces incubated in the presence of insulin, but the decrease was not statistically significant. It was always found that about 50 per cent of the activity of the lower phase was dialysable. The activity left after dialysis of this phase was of the same magnitude in spinal cord pieces incubated in the presence of insulin and in control samples.

DISCUSSION

It has been shown in previous papers (RAFAELSEN, 1958, 1961*a*) that insulin increases the glucose uptake of isolated spinal cord pieces. The results reported here are an attempt to elucidate the fate of the extra glucose taken up. They were mostly negative. The possibility that insulin increased the glycogen synthesis in isolated spinal cord pieces during incubation was first investigated. Glycogen is present in the

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central nervous system only in small amounts. Figures of 0·5–1 mg per g wet brain are recorded. Furthermore, these small amounts of glycogen are very labile. In guinea pig brain slices glycogen falls to 1/10 or less of the original value during the first minutes after isolation, but a synthesis of glycogen takes place in brain slices during incubation (McIlwain and Tresize, 1956). These workers were able to reach glycogen values in the same range as those originally present, and the glycogen synthesized *in vitro* was more stable than that originally present. Synthesis was not stimulated by insulin in the experiments of LeBaron (1955), and only in 1 out of 6 experiments made by McIlwain and Tresize (1956) was a small, but statistically significant increase of glycogen synthesis observed. In isolated rat spinal cord pieces even high insulin concentrations in the medium were without effect on glycogen synthesis. In experiments with incubation of whole isolated frog central nervous systems, Winterstein and Hirschberg (1925) were able to demonstrate that insulin *in vitro* increased the content of glycogen and cerebrosides. This effect of insulin was found both in experiments where the isolated frog central nervous system was incubated in a saline medium without glucose and when glucose was added to the medium. It was remarkable that the increase in glycogen of more than 100 per cent and in cerebrosides of more than 200 per cent was only seen when insulin in 'moderate' concentrations was added to the medium, whereas insulin in high concentrations caused a decrease of both glycogen and cerebroside content. These experiments were later criticized by Kerr (1936) who concluded that the results obtained by Winterstein and Hirschberg were due to an inadequate method for separating cerebrosides from glycogen, to the use of a method for estimating glucose which includes certain non-sugar reducing substances, and to various degrees of post mortem autolysis. This criticism was relevant from a methodological point of view, but from a physiological point of view it is interesting that Winterstein and Hirschberg found an effect on the composition of the isolated frog central nervous system due to insulin even if it is obscure of what the fraction consisted.

It is well known that insulin increases the glycogen synthesis in isolated rat diaphragm during incubation, so that a high insulin concentration in the incubation medium leads to a glycogen content of rat diaphragm muscle after incubation which is about twice as high as that in rat diaphragm muscle incubated without insulin in the medium. In fact, this effect on the glycogen synthesis in rat muscle *in vitro* is the only effect of insulin on the fate of the extra glucose taken up by rat diaphragm which has apparently been found by every worker in this field. We found that insulin added to the medium in a high concentration increased the glycogen synthesis of isolated diaphragms, whereas lower concentrations were unable to increase the glycogen synthesis significantly although 10^{-4} i.u. of insulin/ml in the medium increased the glucose uptake by some 30 per cent (Rafaelsen, 1957). Glucose uptake in isolated rat diaphragm is thus more sensitive to the presence of insulin than is glycogen synthesis.

Lactic acid accumulates in the medium during incubation of brain slices, spinal cord pieces, and diaphragm. An effect of insulin on this accumulation has been reported both with brain slices and diaphragm. Woods, Hunter and Burk (1958) found that aerobic preincubation of mouse brain slices in the presence of insulin $1{\cdot}5 \times 10^{-1}$ i.u./ml caused an increase of glycolysis during subsequent anaerobic incubation. An increase of up to 30 per cent was observed in half of the experiments.

With rat diaphragm SHAW and STADIE (1957, 1959) observed an increase of lactic acid production in the medium in the presence of insulin when a bicarbonate-buffered medium (0·07 M-NaCl, 0·001 M-$CaCl_2$, 0·001 M-$MgCl_2$, 0·05 M-sodium phosphate, and 0·025 M-$NaHCO_3$) was used, but no effect when a phosphate-buffered medium (the above-mentioned medium minus bicarbonate) was applied. In the study reported here, where GEY and GEY's (1936) bicarbonate-buffered medium was used, insulin *in vitro* did not increase the lactic acid accumulation in the medium either in experiments with isolated rat spinal cord, or in experiments with isolated rat diaphragm.

The pentose shunt or hexosemonophosphate pathway is very active in fat tissue and probably does not exist in muscle tissue. It is not known whether this pathway plays any role in the carbohydrate metabolism of the central nervous system. The present results indicate, but do not prove, that the pentose shunt does not function in spinal cord pieces during incubation. No effect of insulin on the conversion of labelled glucose to carbon dioxide was observed. It is noteworthy that in parallel experiments no effect of insulin on the conversion of uniformly-labelled glucose to carbon dioxide by rat diaphragm was found. This negative finding is in accordance with the results of BELOFF-CHAIN *et al.* (1956), but VILLEE and HASTINGS (1949) using the same medium found that insulin increased the proportion of labelled carbon dioxide in experiments with isolated rat diaphragm.

It has been found that galactose and glucose *in vivo* are incorporated in the galactolipids rather quickly (RADIN, MARTIN and BROWN, 1957; BURTON, SODD and BRADY, 1958; MOSER and KARNOVSKY, 1959). It was found in the present experiments that uniformly-labelled glucose was incorporated into lipid fractions *in vitro*, but the relative distribution in different fractions varied from experiment to experiment. In these experiments as in the *in vivo* experiments of RADIN *et al.* (1957), most of the radioactivity extracted by lipid solvents was water soluble and diffusible, i.e. non-lipid. This fraction includes glucose and galactose esters, amino acids, etc., and a moderate increase of activity in this fraction was found in extracts from spinal cord pieces incubated in the presence of insulin. Also in accordance with the results of RADIN *et al.* was the finding that only 1–2 per cent of the water-soluble activity was non-dialysable. This fraction is assumed to consist of the water-soluble glycolipids, and the decrease of activity in this fraction from spinal cord pieces incubated in the presence of insulin was statistically significant.

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SUMMARY

1. As insulin *in vitro* has been found to increase the glucose uptake of isolated rat spinal cord it was investigated as to whether or not insulin influenced (i) the conversion of glucose from the medium to carbon dioxide, (ii) the amount of lactic acid present in the medium after incubation, or (iii) the amount of glycogen present in the spinal cord pieces after incubation. No effect of insulin was found in these experiments.

2. In parallel experiments with isolated rat diaphragm the well-known increase of glycogen content after incubation with insulin in high concentrations was found, but insulin exerted no effect on the conversion of glucose from the medium to carbon dioxide nor on the amount of lactic acid present in the medium after incubation.

3. Lipid extracts were made of spinal cord pieces incubated with radioactive glucose in the absence or presence of insulin. The water soluble non-diffusible fraction,

presumably gangliosides, was 40 per cent lower in spinal cord pieces which had been under the influence of insulin.

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EFFECTS OF ALTERATIONS IN SERUM COPPER LEVELS UPON THE LIVER, BASAL GANGLIA AND CEREBELLAR CORTEX IN THE GUINEA PIG*

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THE presence of copper as a naturally occurring element in whole brain tissue was established by BODANSKY in 1921. Subsequent reports show a relatively high concentration of copper in the globus pallidus, putamen, and caudate nucleus in man (TINGEY, 1937; CUMINGS, 1948).

In the syndrome of Wilson's disease, or hepatolenticular degeneration, large amounts of copper accumulate in these nuclei (GLAZEBROOK, 1945; CUMINGS, 1948; PALMER *et al.*, 1953; CARTWRIGHT *et al.*, 1954). Knowledge of variations in copper concentration in the basal ganglia following alterations in serum copper levels should be of value in understanding the predilection of these nuclei for copper deposition. The purpose of this investigation was to determine: (1) normal copper concentrations in the caudate, amygdaloid, and lenticular nuclei, cerebellar cortex, liver, and serum; (2) changes in copper concentration in these same tissues following high and low copper diets, injections of copper sulphate, and the administration of tranquilizing drugs. The cerebellar cortex was included because of the functional relationship of the cerebellum in the extrapyramidal system. Since the liver functions as a storage organ for copper in vertebrates, liver copper was determined to provide an index to the copper status of the animal.

MATERIAL AND METHODS

General. Adult male and female guinea pigs weighing 500–600 g were used throughout. Glassware was cleaned in 6 N-HCl, followed by exhaustive rinsing with demineralized water, and all solutions were prepared with ion-free water. In each experimental group the procedure was as follows: animals were anaesthetized with 0·3–0·4 ml of an 8% aqueous solution of Evipal (Winthrop) given intraperitoneally; total serum copper was determined before and after each diet and injection series from blood obtained by cardiac puncture; tissue copper concentrations in the liver and the four selected brain regions were determined following sacrifice by viviperfusion with isotonic saline so that no residual blood copper would be present in the tissues analysed. Each liver sample contained tissue from the centre and periphery of a lobe. Cortex from vermis and hemispheres was included in each cerebellar sample. Since the basal ganglia are paired, both nuclei of a pair were analysed as a unit. Because it was impossible to separate the putamen and globus pallidus accurately, these two nuclei were analysed together as the lenticular nucleus. The nuclei were quickly dissected free and placed in tared porcelain crucibles. Wet weights of tissue samples were rapidly determined, and crucibles placed in the drying oven at 110° for 12 hr. After cooling, dry weights were taken, and the samples incinerated in the muffle furnace at 400° for 18 hr as recommended by MCFARLANE (1932). This time and temperature were adequate to produce a completely carbon-free ash.

Chemical determinations. Total serum copper was determined by the method of GUBLER *et al.* (1952) with the following modifications: sodium citrate was omitted and the supernatant fluid was

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diluted to 7 ml and read in 5 cm light path cells at a wave length of 440 mμ on the Beckman spectrophotometer.

The tissue copper method differed from that used for serum copper only in the initial preparation of the material. Following incineration, the ash was taken up in 10 drops of N-HCl and the remainder of the procedure carried out as for serum copper.

Serum and tissue copper standard curves were established. Standard solutions conformed to Beer's law up to a concentration of 3 μg copper/ml in 7 ml. A 'K' value (K = concentration in μg/ml/optical density) of 10·5 was obtained for total serum copper and one of 11·3 for tissue copper.

To determine copper recovery from serum, 2·0, 1·5, 1·0, and 0·5 μg of copper sulphate were added to a series of twelve 1 ml serum samples. Copper recovered ranged from 98·4–100·8 per cent with a mean of 99·9.

Brain copper recovery experiments were carried out by adding 2·0, 1·5, 1·0, and 0·5 μg of copper sulphate to a series of eight 100 mg whole brain homogenate samples. Samples were ashed in the same crucibles used for the experimental tissue incinerations. The range of copper recovered was 97·6–100·9 per cent with a mean of 99·6.

Copper recovery from a series of twelve 100 mg liver homogenate samples was determined by adding 2·0, 1·5, 1·0, and 0·5 μg of copper sulphate and proceeding as in the brain copper recovery method. The range of copper recovered was 99·9–101·1 per cent with a mean of 100·7.

All solutions and drugs used were tested; none gave a positive test for copper.

Animal groups. Eleven groups of experimental animals were used. It was first necessary to establish normal tissue and serum copper levels in the guinea pig. Following this, six groups were employed to determine the effects of high and low copper diets and of the administration of copper sulphate in large and small amounts by intraperitoneal and intracardiac routes. Two tranquilizer drugs, Thorazine (Smith, Kline and French) and Trilafon (Schering) were injected in large and small dosages in four animal groups. The number of control animals used in each group was the same as the number of experimental animals.

Because of its low copper content (KEMMERER *et al.*, 1932), homogenized cow's milk was used for the 16-day low copper diet. A daily supplement of 1·5 mg of ferric chloride, multiple vitamins, and ascorbic acid was added. The 16-day high copper diet was the same except for the daily addition of 5·0 mg of copper sulphate. Control animals received the milk diet with a daily copper supplement of 0·15 mg. The daily copper requirement for the guinea pig was estimated on a weight basis from the daily optimum intake of the rat, 0·05 mg, reported by KEMMERER *et al.* (1932).

The intraperitoneal injections of copper sulphate were given daily for 6 days. In those groups which received intracardiac injections of copper sulphate, animals were sacrificed 15 min following injection. The 0·041 mg amount was calculated to approximately double the serum copper level.

Tranquilizer drugs were given intramuscularly daily for 12 days. In all experimental groups injected with tranquilizers or with copper sulphate, control animals received the same volume of isotonic saline. Animals in all experimental and control groups except those on high and low copper diets were maintained on a diet of rabbit pellets supplemented twice weekly with raw cabbage. Free access to water and salt was allowed.

RESULTS

Results of the normal total serum copper determinations are shown in Table 1.

In normal brains, the lenticular nuclei and cerebellar cortex showed the highest copper concentration (Table 2). The caudate nuclei were lowest in copper content. The most limited concentration range and the lowest copper concentration of all tissues examined was found in the liver. CARTWRIGHT *et al.* (1954) and CUMINGS (1948) have similarly reported some regional brain copper concentrations to be higher than liver copper concentration in man.

To obviate the necessity for additional statistics in evaluating the experimental results which follow, the values in Table 2 may be used as control data, since the ranges and means of all control values obtained in each experimental group do not differ significantly from the normal values presented in Table 2. Probability data were obtained with the 't' method of FISHER.

TABLE 1.—NORMAL TOTAL SERUM COPPER CONCENTRATION

No. of determinations	Range (μg Cu/ml)	Mean (μg Cu/ml)	Standard deviation
50	0·9765–1·5750	1·1525	0·1162

TABLE 2.—NORMAL BRAIN AND LIVER COPPER CONCENTRATIONS

Tissue analysed	No. of animals (nuclei paired)	Mean wet wt (mg)	Mean dry wt (mg)	Copper concentration		
				Range (μg Cu/ 100 mg dry wt)	Mean (μg Cu/ 100 mg dry wt)	Standard deviation
Lenticular nuclei	15	78·3	18·2	4·0–11·6	8·0	2·25
Amygdaloid nuclei	20	29·2	5·2	3·5–6·8	5·5	1·00
Caudate nuclei	15	31·3	6·4	2·5–7·9	5·0	1·70
Cerebellar cortex	15	35·0	7·0	5·0–12·8	8·0	1·92
Liver	15	112·1	33·3	2·4–4·9	3·7	0·85

In response to the 16-day low copper diet, the serum copper fell significantly (Table 3). There was no change in brain copper concentration. Although there was a significant elevation in serum copper of 0·6 μg/ml following the high copper diet (Table 3), there was no rise in brain copper.

The daily intraperitoneal injection of 0·041 mg copper sulphate did not produce a significant rise in serum copper (Table 4). However, the intraperitoneal injection of a massive amount of copper sulphate, 2 mg, resulted in a hypercupremia of 1·9 μg/ml and significant brain copper increases (Table 4). The greatest elevation in brain copper concentration was in the caudate nuclei, followed by the cerebellar cortex and amygdaloid nuclei, in that order. Despite the hypercupremia, there was not a significant increase in lenticular copper.

Fifteen minutes following the intracardiac injection of 0·041 μg of copper sulphate, the caudate nucleus again showed the largest uptake of copper while the cerebellar increase was again less (Table 5). In this group, the first significant rise in lenticular copper was found. This lenticular increase exceeded the rise in amygdaloid copper. In response to the intracardiac injection of 2 mg copper sulphate, the greatest concentration was again found in the caudate nuclei and cerebellar cortex, followed by the amygdaloid nuclei (Table 5). The increment in lenticular copper was significant but it is striking that this 2·4 μg increase is less than half that found in the other brain regions, even in the presence of this massively elevated serum copper level.

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TABLE 3.—EFFECT OF LOW AND HIGH COPPER DIETS ON TISSUE COPPER LEVELS

Diet	No. of animals (nuclei paired)	Tissue analysed	Copper concentration* (μg Cu/100 mg dry wt)		Standard deviation	Probability (*P*)
			Range	Mean		
Low copper	9	Lenticular nuclei	5·6–10·0	7·8	1·21	0·6–0·8
		Amygdaloid nuclei	4·1–6·6	5·3	0·69	0·4–0·6
		Caudate nuclei	4·5–7·5	5·8	0·79	0·4–0·6
		Cerebellar cortex	5·7–11·1	8·0	1·53	>0·8
		Liver	2·0–3·1	2·4	0·38	<0·01
		Serum	0·9660–1·0290	1·0045	0·06	<0·01
High copper	8	Lenticular nuclei	5·8–9·6	8·0	1·06	>0·8
		Amygdaloid nuclei	4·1–6·2	5·5	0·65	0·6–0·8
		Caudate nuclei	4·5–8·0	6·0	0·89	0·2–0·4
		Cerebellar cortex	6·0–9·8	7·8	1·15	>0·8
		Liver	4·6–7·8	6·0	0·87	<0·01
		Serum	1·3125–1·9328	1·7116	0·07	<0·01

* Serum copper concentration in Tables 3 through 7 are expressed in μg/ml..

Results of the injections of Thorazine (Table 6) and Trilafon (Table 7) show a significant serum copper rise of 0·6 μg/ml in each group which is independent of the dosage or drug administered. As in the copper sulphate injected groups, the caudate nuclei and cerebellar cortex consistently showed significant copper increments. However, there was never a significant rise in amygdaloid or lenticular copper in any of the four experimental groups receiving tranquilizers. Liver copper concentration increased significantly in each group, the elevations being independent of dosages or drugs.

DISCUSSION

Of the four brain regions examined, the caudate nuclei and cerebellar cortex consistently showed the largest increases in copper concentration in all experimental

TABLE 4.—EFFECT OF INTRAPERITONEAL INJECTIONS OF COPPER SULPHATE ON TISSUE COPPER LEVELS

Injection	No. of animals (nuclei paired)	Tissue analysed	Copper concentration* (μg Cu/100 mg dry wt)		Standard deviation	Probability (P)
			Range	Mean		
0·041 mg $CuSO_4$	10	Lenticular nuclei	6·8–10·6	8·4	1·01	0·4–0·6
		Amygdaloid nuclei	3·9–6·6	5·3	0·79	>0·8
		Caudate nuclei	4·0–6·3	5·5	0·62	0·4–0·6
		Cerebellar cortex	6·9–9·5	8·4	0·79	0·4–0·6
		Liver	3·1–5·0	4·1	0·47	0·02–0·05
		Serum	1·0500–1·3650	1·2138	0·07	0·2–0·4
2·0 mg $CuSO_4$	10	Lenticular nuclei	7·2–10·1	8·9	0·90	0·05–0·1
		Amygdaloid nuclei	5·1–7·5	6·5	0·67	<0·01
		Caudate nuclei	5·7–10·0	8·4	1·38	<0·01
		Cerebellar cortex	8·3–12·9	10·7	1·36	<0·01
		Liver	16·4–25·9	21·7	3·29	<0·01
		Serum	2·8350–3·4650	3·1217	0·17	<0·01

groups in which a rise in brain copper was found. Except in those groups which received tranquilizers, the copper elevation in these two brain tissues roughly paralleled the rises in serum copper. Since the serum copper increase of 0·6 μg/ml produced by the high copper diet did not elicit a change in brain copper, it would seem that hypercupremia of greater magnitude must be necessary to result in copper uptake by the brain. Opposed to this, however, is the fact that injections of tranquilizers resulted in a rise in serum copper of identical magnitude. Following the injection of all doses of both tranquilizers used, the caudate nuclei and cerebellar cortex—and no other brain regions examined—showed increases in copper concentration comparable in amount to those found in other experimental groups in which the serum copper rise was as much as three times greater than 0·6 μg/ml. In these latter groups, lenticular and amygdaloid copper elevations were found, yet these two nuclei demonstrated no rise in copper content in response to the drug-induced hypercupremias. It seems

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TABLE 5.—EFFECT OF INTRACARDIAC INJECTIONS OF COPPER SULPHATE ON TISSUE COPPER LEVELS

Injection	No. of animals (nuclei paired)	Tissue analysed	Copper concentration* (μg Cu/100 mg dry wt)		Standard deviation	Probability (P)
			Range	Mean		
0·041 mg $CuSO_4$	18	Lenticular nuclei	7·2–11·4	9·5	1·08	0·01–0·02
		Amygdaloid nuclei	5·1–7·6	6·4	0·67	0·01–0·02
		Caudate nuclei	5·3–8·1	7·0	0·88	0·02–0·05
		Cerebellar cortex	7·0–11·6	9·5	1·75	0·01–0·02
		Liver	3·7–5·8	4·4	0·52	<0·01
		Serum	2·3100–2·4990	2·4113	0·07	<0·01
2·0 mg $CuSO_4$	18	Lenticular nuclei	8·0–12·2	10·4	1·08	<0·01
		Amygdaloid nuclei	5·8–17·2	11·2	2·69	<0·01
		Caudate nuclei	6·0–17·8	11·5	3·66	<0·01
		Cerebellar cortex	11·3–17·6	15·6	2·14	<0·01
		Liver	5·2–14·5	10·6	2·50	<0·01
		Serum	All read at 2·0 optical density or 0 per cent transmission			

reasonable that one cannot equate the tissue responses to hypercupremias produced by administration of copper sulphate by various routes to tissue responses to the hypercupremias elicited by the tranquilizers. In the former, copper was given directly to the animal; in the latter, the source of the copper increase is unknown, since no copper was found when the two tranquilizers were tested for the ion. Therefore, it must be concluded that the source of the copper increase in serum, brain, and liver is endogenous. The possibility of an effect of the drugs upon the liver resulting in a release of storage copper into the bloodstream is negated by the elevations in liver copper. Thus it is possible to theorize that serum copper may have risen first and brain and liver copper increases were sequential. The serum copper rise in response

TABLE 6.—EFFECT OF THORAZINE ON TISSUE COPPER LEVELS

Dosage	No. of animals (nuclei paired)	Tissue analysed	Copper concentration* (μg Cu/100 mg dry wt)		Standard deviation	Probability (*P*)
			Range	Mean		
1·2 mg	10	Lenticular nuclei	6·4–9·9	8·1	0·82	0·6–0·8
		Amygdaloid nuclei	4·8–5·7	5·3	0·23	0·6–0·8
		Caudate nuclei	6·3–7·9	7·2	0·44	<0·01
		Cerebellar cortex	9·0–11·6	10·5	0·82	<0·01
		Liver	3·8–4·7	4·4	0·33	<0·01
		Serum	1·3870–1·8060	1·7048	0·12	<0·01
25·0 mg	10	Lenticular nuclei	6·4–9·0	7·9	0·87	0·6–0·8
		Amygdaloid nuclei	4·2–6·6	5·3	0·70	0·4–0·6
		Caudate nuclei	5·7–8·0	7·3	0·75	<0·01
		Cerebellar cortex	7·2–12·8	10·0	1·53	<0·01
		Liver	3·8–5·7	4·7	0·59	<0·01
		Serum	1·3870–1·9005	1·7086	0·55	<0·01

to administration of tranquilizers might be attributed to possible increased absorption of copper from the gastrointestinal tract and/or the release of copper from cells of other body tissues, brought about by some pharmacological action of the drugs upon cell membranes and cell constituents, This facet of the investigation has not been pursued. There is the possibility of a greater specificity of this theoretical pharmacologic action for the cerebellar cortex and caudate nuclei, rendering these two tissues more receptive to the uptake of copper.

The amygdaloid nuclei were less responsive, in terms of increased copper uptake, to the hypercupremias produced by tranquilizers and by direct administration of copper sulphate than the cerebellar cortex and caudate nuclei.

The response of the lenticular nuclei to the experimental hypercupremias was markedly different from that of the other brain regions examined; a massive elevation of the serum copper level by intracardiac injection of 2·0 mg of copper sulphate resulted in a rise in lenticular copper of 2·4 μg, but when the serum copper level was

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TABLE 7.—EFFECT OF TRILAFON ON TISSUE COPPER LEVELS

Dosage	No. of animals (nuclei paired)	Tissue analysed	Copper concentration* (μg Cu/100 mg dry wt)		Standard deviation	Probability (*P*)
			Range	Mean		
1·2 mg	10	Lenticular nuclei	6·8–9·4	8·0	0·81	0·6–0·8
		Amygdaloid nuclei	3·8–6·9	5·6	1·00	0·6–0·8
		Caudate nuclei	5·7–9·0	7·4	0·99	<0·01
		Cerebellar cortex	8·3–12·8	10·3	1·31	<0·01
		Liver	3·5–4·7	4·3	0·41	0·01–0·02
		Serum	1·2180–1·9005	1·7050	0·22	<0·01
25·0 mg	10	Lenticular nuclei	6·3–9·0	8·0	0·79	>0·8
		Amygdaloid nuclei	4·6–6·1	5·4	0·51	0·6–0·8
		Caudate nuclei	6·4–8·0	7·3	0·48	<0·01
		Cerebellar cortex	8·3–11·3	10·0	0·79	<0·01
		Liver	3·8–4·8	4·4	0·32	0·02–0·05
		Serum	1·3250–1·8505	1·7100	0·19	<0·01

increased by 1·5 μg/ml following the intracardiac administration of 0·041 mg of copper sulphate, the concentration of copper in this nucleus increased by 2·1 μg. Furthermore, a serum copper rise of 1·9 μg/ml, produced by intraperitoneal injection of 2·0 mg of copper sulphate, did not elicit a significant elevation in lenticular copper. It is evident that copper uptake by these nuclei does not, unlike that of the other three brain regions, parallel the changes in serum copper concentration. It seems possible that an optimum serum copper level may exist which is conducive to a greater uptake of copper by these nuclei.

A consideration of the routes of administration of copper sulphate as possible factors influencing the amount of copper absorbed by the lenticular nuclei reveals that they showed a rise in copper concentration only in those groups in which copper was given by the intracardiac route. However, the response of other brain regions to the hypercupremia induced by intraperitoneal administration of 2·0 mg of copper sulphate by elevations in copper concentration indicates that copper given by this

route was not changed in some manner so that a direct-reacting fraction of serum copper was no longer available for passage across cell membranes.

There is one other possibility to be considered in evaluating the selective uptake of copper by the lenticular nuclei: the lenticular copper determinations represent copper concentrations in two different nuclei, the putamen and globus palladius. Although these are both nuclei of the extrapyramidal motor system, their histological and functional patterns differ and their metabolic requirements might be expected to differ also. If each nucleus varies in its requirements and absorptive properties for copper, the analysis of both as a unit might yield results which are actually representative of neither.

Results of the liver copper determinations in each experimental group indicate that this organ is capable of storing comparatively large amounts of administered copper. The decrease of 1·3 μg in liver copper concentration found in the low copper diet group at the end of the 16-day diet period suggests that there may be a labile fraction of liver copper which is not capable of maintaining serum copper at a normal level for this time.

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SUMMARY

Copper sulphate-induced hypercupremia of 0·6 μg/ml was not adequate to produce copper increments in the brain regions examined. In response to serum copper rises above this level, the caudate nuclei and cerebellar cortex consistently showed the largest uptake of copper, the uptake paralleling the serum copper elevations.

The failure of increases in lenticular copper to follow rises in serum copper suggests the existence of an optimum level of serum copper to which these nuclei are most responsive. Uptake of copper by them was consistently less than that of the caudate nuclei and cerebellar cortex.

Following tranquilizer-induced hypercupremias of 0·6 μg/ml, copper increased in only two brain tissues—the caudate nuclei and cerebellar cortex.

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THE CEREBRAL DEFECT IN TAY-SACHS DISEASE AND NIEMANN-PICK DISEASE*

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THE nature of the functional and anatomic disturbance in the central nervous system which occurs in the constitutional syndromes referred to as 'lipidoses' has not been explained. There has been an understandable tendency to assume that the same hereditary defection which produces an accumulation of lipid in the solid viscera of these patients is also responsible for the changes in nervous tissue. This is the traditional view (HSIA, 1959), although THANNHAUSER (1953), in a penetrating review of the problem, raises important doubts about the certainty of this conclusion.

Descriptions of Niemann-Pick disease have offered major support to the concept of the unitarian hypothesis for brain and visceral lipid derangement, since the classical patients with this diagnosis invariably had cerebral handicaps and many of the early studies showed brain sphingomyelin increases (KLENK, 1954). In Tay-Sachs disease, the failure to find ganglioside accumulations in liver and spleen was explained by the probable absence, in these regions, of a significant natural synthetic locus. In chronic Gaucher's disease, it was stated (THANNHAUSER, 1953) that the cerebroside defect is not fully manifest until the critical period of cerebral maturation is completed (and thus the brain is spared). These generalizations are found to be unsatisfactory, however, as more patients receive careful study. Furthermore, the existing published reports on brain lipid analysis in children with lipidosis problems are difficult to summarize because of the small number of patients, and the differences among authors in clinical classification, preparation of specimens for analysis, chemical techniques employed, and method of recording results. It therefore seems to be of value to present recent material from this hospital which bears on the thorny problem of the type of nervous system handicap found in certain of the lipidoses.

MATERIAL AND METHODS

The patients in this study are from The Children's Hospital Medical Center in Boston, except as noted. The diagnoses were supported by major clinical and pathologic examinations. Chemical analyses of the tissues were performed in schemes which 'screened' for abnormalities, and did not follow exhaustive purification techniques and structural measurements. These methods have been discussed, and their sources given, in a previous review (CROCKER and FARBER, 1958). In brief, careful dissection was performed to obtain discrete gray and white matter portions, the tissue was treated with 2:1 chloroform–methanol, and the extract washed by the original FOLCH double-beaker technique; lipid phosphorus was measured with KING's reagent, sphingomyelin by the 1946 SCHMIDT method,

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cholesterol by the SCHOENHEIMER-SPERRY procedure, and the total lipids were assayed gravimetrically. In addition, an estimate of glycolipids was made by use of gentle overnight hydrolysis of the lipid extract solids with N-HCl at 100°, followed by determination of hexose (FOLIN-SVEDBERG reducing substances method) with a galactose standard, the principles presented by BOGOCH (1958) being used. All specimens were analysed fresh, or, more commonly, after brief frozen storage.

The exposure of a chloroform–methanol extract of brain tissue to a water interface allows the partition of the lipids into two phases. Those which pass into the upper phase are, loosely speaking, the water-soluble fraction and will be referred to in this report as the 'aqueous phase' lipids. Those remaining in the chloroform or lower phase are predominantly water-insoluble and can be identified as the 'washed' lipids, or 'non-aqueous phase' lipids. It was assumed that the principal glycolipid of this latter phase is of the cerebroside type, and a mean carbohydrate content of 20 per cent was taken for the calculation.

RESULTS

Normal children

To establish standards for the evaluation of the pathologic material, cerebral specimens were analysed from a group of 16 children, from 2 days to 8 years of age, who had died of non-neurologic illnesses and had no anatomic disease of the brain (Fig. 1 and Table 1). Other studies of this sort have been reported by BRANTE (1949),

TABLE 1.—ANALYSIS OF CEREBRAL LIPIDS IN NORMAL CHILDREN, PERFORMED ON WASHED CHLOROFORM–METHANOL EXTRACTS

Age period	No. of patients	Total lipids	Phospholipids	Sphingomyelin	Glycolipids
		(Per cent of fresh weight)			
	Gray matter				
Newborn	2	3·1–5·0	2·0–2·6	0·3–0·6	0·2–0·4
First year	7	4·3–5·8	2·2–3·6	0·4–0·8	0·4–0·7
After 1 year	7	4·7–6·7	2·6–3·7	0·4–0·8	0·4–0·8
Notes: a. Sphingomyelin 16–25 per cent of total phospholipids b. Glycolipids 6–15 per cent of total lipids c. Cholesterol levels from BRANTE (1949)—newborn: 0·4–0·5; first year: 0·4–0·6; later: 0·6–0·9					
	White matter				
Newborn	2	3·8–6·5	2·1–2·3	0·4–0·6	0·1–0·2
First year	7	4·4–12·1	2·2–5·5	0·5–1·3	0·2–2·8
(Sharp increases occur during this period)					
After 1 year	7	9·9–18·0	4·6–7·1	1·1–2·7	1·3–3·7
Notes: a. Sphingomyelin 16–38 per cent of total phospholipids b. Glycolipids 11–23 per cent of total lipids c. Cholesterol levels from BRANTE (1949)—newborn: 0·4–0·6; first year: 1·3–1·4; later: 2·6–4·5					

JOHNSON *et al.* (1949), and CUMINGS *et al.* (1958). We did not routinely perform cholesterol measurements, but have found the values of BRANTE to be representative. The results are reported as 'per cent of fresh weight', although it is acknowledged that this unit expression masks the water-content rearrangements which occur in the brain of the maturing infant. There are available in other articles (BRANTE, 1949; CUMINGS *et al.*, 1959), a number of lists of contents of solids in the brain at various ages, from which conversions can be made. The valuable reviews of SPERRY (1955, 1960) give a complete survey of early chemical changes in normal brains.

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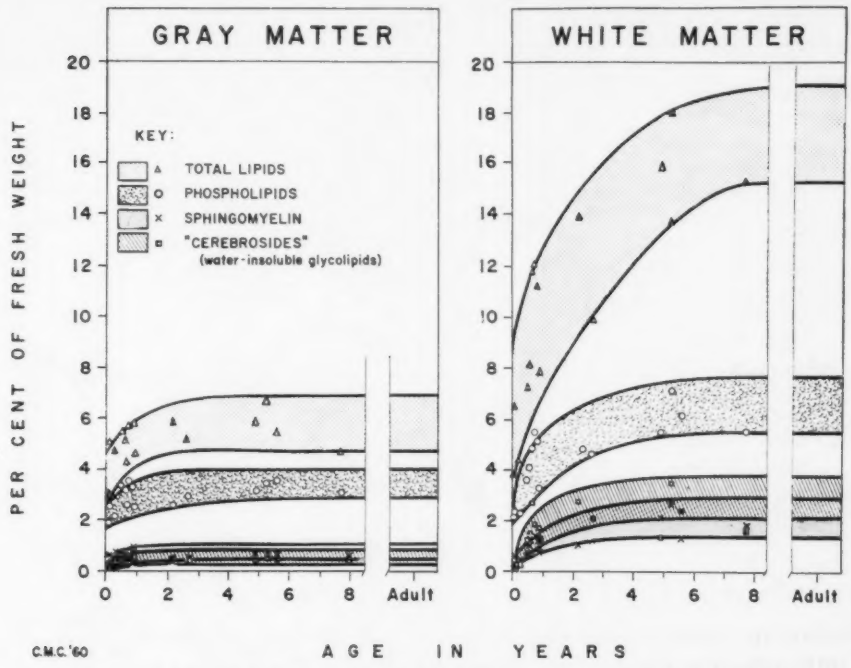

FIG. 1.—Changes in brain lipid levels with increasing age, for 16 children dying of non-neurologic illnesses.

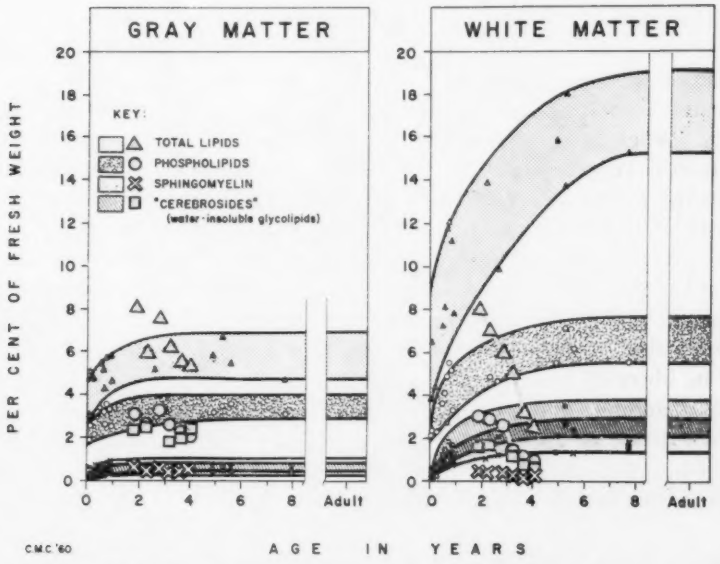

FIG. 2.—Lipid analysis of cerebral specimens in 6 children with Tay-Sachs disease, superimposed on the normal lipid maturation pattern of the brain as determined by the same techniques.

TABLE 2.—ANALYSIS OF CEREBRAL LIPIDS IN CHILDREN WITH TAY-SACHS DISEASE, PERFORMED ON WASHED CHLOROFORM–METHANOL EXTRACTS

Patient	Sex	Age (Yr.)	Total lipids	Phospho-lipids	Sphingo-myelin	Cholesterol	Glycolipids
			(Per cent of fresh weight)				
Gray matter							
D.R.	M	1 11/12	8·03	3·10	0·56	0·47	2·31
S.K.	M	2 3/12	5·97	2·50	0·45	0·74	2·49
D.St.	F	2 10/12	7·50	3·29	0·60		
M.O.	M	3 2/12	6·18	2·58	0·42	0·83	1·84
D.Sh.	M	3 9/12	5·55	2·23	0·56	0·64	2·14
B.M.	M	4 0/12	5·45	2·01	0·74	0·64	2·44
White matter							
D.R.	M	1 11/12	7·98	3·22	0·57	0·70	1·72
S.K.	M	2 3/12	7·00	2·95	0·57	0·75	1·75
D.St.	F	2 10/12	6·10	2·67	0·52		
M.O.	M	3 2/12	5·07	1·61	0·32	0·62	1·35
D.Sh.	M	3 9/12	3·29	1·25	0·29	0·33	0·85
B.M.	M	4 0/12	2·74	1·21	0·44	0·46	0·68

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Tay-Sachs disease

Cerebral specimens were available from six children with the characteristic type of infantile amaurotic family idiocy (Tay-Sachs disease), spanning the usual age range of expiration in this disease (2–4 years). The lipid analyses display a remarkably uniform group of abnormalities (Fig. 2 and Table 2).

In *gray matter*, the phospholipid (including sphingomyelin) and cholesterol levels show no consistent deviations from normal, but the non-aqueous phase glycolipid assay demonstrates, in all instances, a major increase, with a concomitant upper normal or mildly elevated total lipid figure. As could have been expected, the analysis of lipid-conjugated carbohydrate in the washed extract incompletely demonstrates the abnormality. When analyses were run simultaneously on a portion of the same gray matter extract not exposed to a water interface, the total lipid content was found to be higher to the extent of 1·6–2·6 (average 2·2) per cent of fresh weight. This increase could be almost entirely accounted for by aqueous phase glycolipid. This phenomenon is documented in Table 3 where it is shown that nearly as great an additional 'lipid hexose' content is found in the aqueous phase as was present in the non-aqueous phase. With the data available, it is impossible to convert reducing-substance analysis directly to ganglioside content since hexosamine-derived reducing groups would be liberated by the N-HCl hydrolysis employed and no coordinate neuraminic acid determinations were performed. It is possible that in this routine type of washed extract, an incomplete removal of the greatly elevated ganglioside fraction occurred. Alternatively, one could conclude that *both* aqueous and non-aqueous phase glycolipid gray matter increases are found in Tay-Sachs patients' specimens.

Almost startling inadequacies of lipid are found in the *white matter*. Particularly impressive is the regularly decreasing lipid content as survival is prolonged. In the final phases of this disease, the cholesterol level diminished to one-ninth or one-tenth

TABLE 3.—'LIPID HEXOSE' MEASUREMENTS IN CHLOROFORM–METHANOL EXTRACTS OF CEREBRAL CORTEX

Patient	Age	LIPID HEXOSE 'Washed extract' (non-aqueous phase) (mg per 100 g of fresh weight)	Additional increment if unwashed (mg per 100 g of fresh weight)
Normal			
G.R.	6 days	72	64
G.L.	8 months	106	62
S.O.	5 3/12 yr.	130	64
			Mean 63
Niemann-Pick disease			
R.N.	1 9/12 yr.	160	170
C.A.	3 3/12 yr.	159	92
			Mean 131
Tay-Sachs disease			
D.R.	1 11/12 yr.	462	560
S.K.	2 3/12 yr.	498	326
M.O.	3 2/12 yr.	368	504
D.Sh.	3 9/12 yr.	428	366
B.M.	4 0/12 yr.	488	338
			Mean 419

of the expected figure for age, and non-aqueous phase glycolipids to about one-fourth of normal. Phospholipid content is also about one-fourth of normal in the older Tay-Sachs patients. The results (Table 2) have usually been from analysis of frontal lobe specimens; in those instances where portions of occipital lobes were simultaneously extracted, the white matter was slightly less deficient in two patients (*B.M.* and *M.O.*) and somewhat poorer in another (*D.R.*).

TABLE 4.—COMMON CLINICAL FORMS OF NIEMANN-PICK DISEASE

Enlargement of liver and spleen	Chest X-ray	Onset of CNS symptoms	Elevation of lipids Blood	Elevation of lipids Tissue	Age at death (Yr.)	Ethnic background
Group A. 'CLASSICAL' NIEMANN-PICK DISEASE (6 families)						
++++	++	Early infancy (often CRS)	++	++++	1–2	Often Jewish
Group B. HEAVY VISCERAL INVOLVEMENT, NORMAL CNS (3 families)						
++++	++	None seen at 4–13 yrs.	++	++++	—	Mixed
Group C. MODERATE COURSE (5 families)						
++	0	Late infancy	0	++	3–5	Non-Jewish
Group D. NOVA SCOTIAN GROUP (4 families)						
Usually +++	0	Early to middle childhood	0	++	12–20	Catholic

Niemann-Pick disease

The clinical picture in Niemann-Pick disease, as the syndrome is conventionally defined, is variable. The cumulative experience in this hospital, with patients who show a constitutional affliction characterized by major visceral sphingomyelin accumulation in the usual type of 'foam cell' is summarized in Table 4. There, four

categories have been selected (groups 'A'–'D') to aid in the study of clinical and chemical data. The more detailed review (CROCKER and FARBER, 1958) should be consulted for illustration of most of these patients. Cerebral specimens have become available in each of these groups where death has occurred from the crippling advance of the disease (Fig. 3, and Table 5). The abnormalities are less marked and less

TABLE 5.—ANALYSIS OF CEREBRAL LIPIDS IN CHILDREN WITH NIEMANN-PICK DISEASE, PERFORMED ON WASHED CHLOROFORM-METHANOL EXTRACTS

Patient	Sex	Age (Yr.)	Total lipids	Phospho-lipids	Sphingo-myelin	Cholesterol	Glycolipids
			(Per cent of fresh weight)				
	Gray matter						
	Group A						
R.N.	M	1 9/12	6·90	4·33	1·62	0·94	0·80
	Group C						
C.A.	M	3 3/12	5·18	2·94	0·50	0·73	0·80
V.F.	F	3 3/12	6·26	2·89	0·52	1·27	1·01
L.G.	F	5 3/12	4·23	2·72	0·32	0·76	0·54
	Group D						
E.D.	M	19 1/12	6·65	3·64	0·76	0·52	1·17
	White matter						
R.N.	M	1 9/12	8·68	4·43	1·59	1·22	1·19
C.A.	M	3 3/12	11·5	4·52	1·00	1·42	2·46
V.F.	F	3 3/12	11·8	4·72	1·18	1·92	2·13
L.G.	F	5 3/12	5·39	1·90	0·40	1·42	0·70
E.D.	M	19 1/12	18·7	8·45	1·96	2·47	5·19

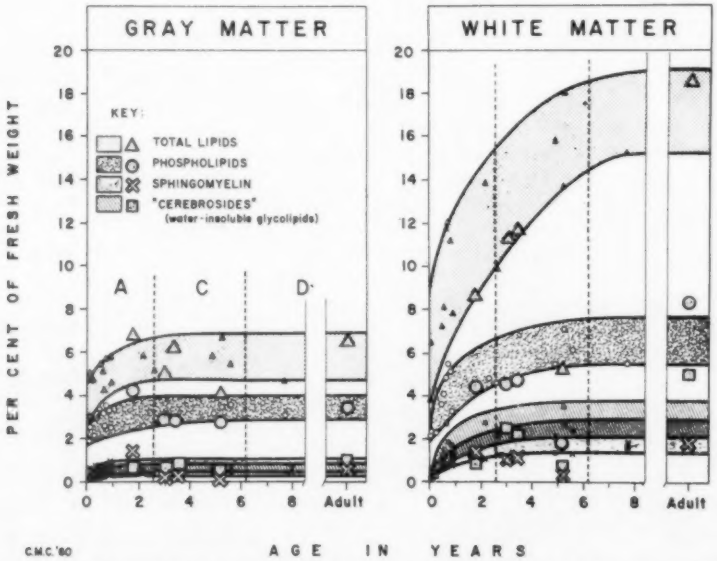

FIG. 3.—Cerebral lipid analyses in 5 patients with Niemann-Pick disease, compared with expected lipid levels for age. (Groups A, C and D).

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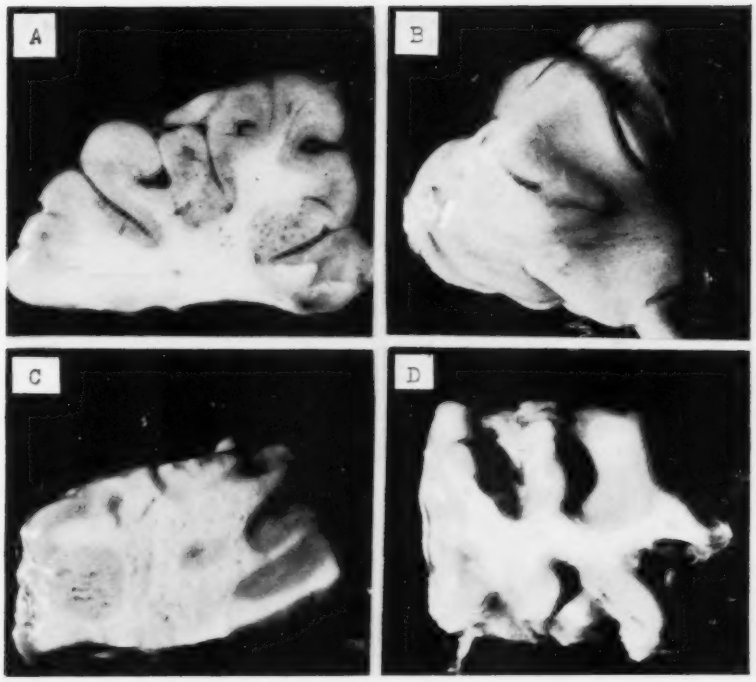

FIG. 4.—Gross appearance of pieces of unfixed cerebrum in various constitutional syndromes.

A. *Tay-Sachs disease*, patient *S. K.*, age 2 3/12 years, brain weight 1560 g (normal for age 1084). Gray matter conspicuous and heavy-appearing, with white matter still fairly well preserved (see Table 2, for chemical analyses).

B. *Tay-Sachs disease*, patient *D. Sh.*, age 3 9/12 years, brain weight 1632 g (normal 1181). Gray matter now stands out as firm 'ribbon' with white matter soft, receding, and partially cystic.

C. *Niemann-Pick disease*, patient *C. A.*, age 3 3/12 years, brain weight 950 g (normal 1153). White matter is firm and well-formed, while the gray matter is soft and reduced in volume, with wide sulci.

D. *Hurler's disease*, with mental retardation, patient *F. P.*, age 12 8/12 years, brain weight 980 g (normal 1360). Gross brain changes resemble those in Niemann-Pick disease, with white matter standing out in firm relief, and gray matter reduced and soft in texture. Chemical analysis yielded normal results for gray matter, and slightly lowered white matter lipids (except glycolipids).

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consistent than those found in the Tay-Sachs children. The chemical studies suggest that a group of 'diseases', rather than a single disorder, is being included under the existing diagnostic terminology. Whereas the Tay-Sachs brain is overweight for age with abnormally firm gray matter and degenerated white matter, the brain of the Niemann-Pick child is underweight and shows a soft cortex (Fig. 4).

In only one instance (patient *R.N.*) is the sphingomyelin level of *gray matter* increased. This child died in infancy and showed all of the 'classical' features of Niemann-Pick disease as it was originally described (including macular degeneration). It is probable that most of the early published reports on brain analysis in this syndrome were derived from this type of patient. The other children in our series had normal cortical sphingomyelin assays but their visceral levels for this material were unmistakably abnormal (Table 6). The cholesterol analyses showed normal or

TABLE 6.—COMPARISON OF VISCERAL AND CEREBRAL SPHINGOMYELIN LEVELS IN NIEMANN-PICK DISEASE

Patient*	SPLEEN (if available) Sphingomyelin (% Fresh wt)	Age (Yr.)	CEREBRAL GRAY MATTER Sphingomyelin (% Fresh wt)	Histologic Changes	Age at death (Yr.)
Group A. SEVERE, EARLY CNS DETERIORATION					
R.N. (7)	4·57	1·1	1·62	Severe	1·8
B.L.	4·54 (Liver)	0·5			2·1
Group B. APPARENTLY NORMAL CNS FUNCTION					
J.K.	6·29	3·3		No problem at 4·0 years	
G.K. (17)	7·0	2·1		No problem at 11·5 years	
S.S. (18)	6·32	11·0		No problem at 13·5 years	
Group C. MODERATE CNS PROBLEMS, SLOWER COURSE					
C.A. (8)	1·25	3·0	0·50	Irregular	3·2
V.F. (9)	1·65	2·9	0·52	Moderate	3·2
I.P. (10)	2·27	0·7			3·8
L.G. (12)	1·05 (Lymph node)	5·2	0·32	Moderate	5·2
Group D. LATE, EVENTUALLY SEVERE, CNS DEFECT					
E.D. (16)	0·57 (Lymph node)	19·1	0·76	Moderate	19·1

* Identifying number in previous review (CROCKER and FARBER, 1958).
(G.K. and I.P. specimens analysed by Dr. L. L. UZMAN)

mildly increased results. Non-aqueous phase glycolipid levels also tended toward mild elevation, although not approaching the magnitude of the increase seen in Tay-Sachs disease. There was a mild augmentation of the aqueous phase glycolipid as well (Table 3).

There are also relatively mild abnormalities found in the *white matter*. Patient *L.G.* showed uniformly deficient results somewhat similar to those found in Tay-Sachs disease. Patient *E.D.*, whose long survival was most unexpected by his early observers, proved to have completely adequate brain lipid results. The other children were near normal, although it is interesting to note a seemingly consistent mild depression of the cholesterol content.

DISCUSSION

Analyses of normal brain

The white matter analyses show that after about three months of age the human cerebral hemispheres undergo a dramatic increase in non-aqueous phase glycolipid

content (a ten-fold implementation, based on wet weight figures, in less than a year). Judging from the report of BRANTE (1949), the cholesterol content of white matter shows a similar rise, although not as extreme. The phospholipid increases are gradual, being more notable for degree in the sphingomyelin fraction (final four-fold rise). Because a parallel increase in stainable myelin appears in this same early age group, it is customary to refer to cerebrosides, cholesterol, and sphingomyelin, as the 'myelin lipids', or 'sheath-typical lipids'.

Detailed reports on the maturation of brain lipids are available from mouse (FOLCH-PI, 1955), and rabbit (DAVISON and WAJDA, 1959) studies, with similar findings to those reported for humans, although these projects have been handicapped by the need to utilize whole brain instead of white and gray matter. Some recent reports have questioned whether or not the fractions isolated as sphingomyelin (EDGAR and SMITS, 1959) and glycolipid (UZMAN and RUMELEY, 1958) in these developing animal brains were typical.

The brain in Tay-Sachs disease

Historical. It is difficult to utilize previously published studies on Tay-Sachs brains for comparison with the results reported here. Most of the earlier reports have been on whole brain assays, with results given as per cent of dry weight (see review of FROEHLICH, 1957). CUMINGS (1957) has presented analyses on several patients, also showing elevated gray matter cerebroside and ganglioside levels. FOLCH-PI and LEES (1959) performed assays on one of the same brains reported here (patient *D. St.*), listing a ganglioside level of 1·46 per cent in gray matter (and also reporting water content figures). KLENK's pioneering reports on this subject are well known, and are responsible, of course, for the first knowledge of the ganglioside increase in this disease. A valuable study by TINGEY (1959) gives data showing that cortical levels are increased for all fractions: lipid galactose, neuraminic acid, and hexosamine. In a histochemical review, DIEZEL (1957) discusses the positive PAS-staining of the abnormal cortical neurones of Tay-Sachs disease in frozen sections, with fading of the reaction after paraffin embedding. LANDING and FREIMAN (1957); and NAKAI and LANDING (1960) have also described a weak PAS-positivity for tissue in paraffin sections. The intense deterioration of the white matter in this disease is well illustrated in the reports of ARONSON (1954) and ARONSON *et al.* (1955).

Data of the present study. The discreteness, uniformity, and magnitude of the *increase in the gray matter glycolipids* in the patients with Tay-Sachs disease are striking and suggest that this metabolic alteration is a basic one. The results are consistent, in spite of the histologic evolution taking place in this age period of the disease. In the conventional type of analysis reported here, the increase in lipid hexose appears in both the non-aqueous and aqueous phases of extract, but the increase in the water-soluble fraction is more unusual. This is in obvious agreement with the oft-reported ganglioside surfeit in these brains. The uniformity of the analytic results, in spite of the considerable variation in age, clinical handling, and ethnic background of the patients, is reminiscent of the notable constancy of the abnormal spleen cerebroside level across a wide age group reported recently in Gaucher's disease (CROCKER and LANDING, 1960). In both instances, a reasonable theory of pathogenesis must await refinement of knowledge about the normal turnover mechanisms for these biologic elements.

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The *white matter deterioration* is of great interest. For final interpretation, a detailed correlation must be made with the exact histologic situation, but some preliminary comments are justified.

Because of the serial nature of the ages of our Tay-Sachs patients, it is possible to envisage a progressive disease of white matter. The younger patients show only moderate chemical defect at an age when the most critical myelin formation should already have been established. This suggests that primary myelin formation was not seriously affected. The white matter glycolipid (non-aqueous phase) level remains within the normal range long after the other lipids have become significantly reduced. It will require sampling at still younger ages to test the possibility that the early situation might be that of a glycolipid increase in this region as well. Poor maintenance of myelin does indeed occur as the Tay-Sachs baby ages, but this is not to imply that there is a correspondence in mechanism to the standard 'Wallerian' demyelination. In this, sphingomyelin and cerebrosides are lost most rapidly, with the total cholesterol level falling slowly (JOHNSON *et al.*, 1950; MCCAMAN and ROBINS, 1958), the converse of the situation found in the Tay-Sachs white matter. Furthermore, the expected appearance of cholesterol esters has not been found (CUMINGS, 1956). The intriguing studies currently being reported on the white matter biochemistry in the 'leukodystrophies' suggests that this tissue may have a more dynamic metabolism than had been the conventional view. The sulphatide increase found by AUSTIN (1960) in white matter in the metachromatic form of diffuse cerebral sclerosis, is a good example.

The brain in Niemann-Pick disease

Historical. The early reports of brain lipid assay in Niemann-Pick disease are summarized by BARTSCH (1957), where sphingomyelin increases (? cortex only) have been stated to be routine. Results reported by CUMINGS (1957) from an eighteen month child show an elevated gray matter sphingomyelin with normal or slightly increased white matter phospholipids. Both KLENK (1954) and CUMINGS (1957) present increased neuraminic acid levels in the cortex, although in the report of TINGEY (1959) this would appear to be normal. THANNHAUSER (1953) could not demonstrate brain sphingomyelin increases in this disease.

Data of the present study. When the whole group of patients qualifying for the diagnosis of Niemann-Pick disease is examined collectively, it is seen that the brain chemical changes are not specific or uniform. The previous conclusion that the *gray matter sphingomyelin level* is elevated appears to have been derived from the analysis of the 'classical 'type of patient only, and for this picture (see patient *R.N.*) we would concur. In the other children, major histologic alterations in the cortex are not reflected in phosphatide elevations in gray matter.

There is an old and ever-recurring hypothesis that Tay-Sachs disease is merely the Niemann-Pick anomaly restricted to the nervous system (see discussion in CROCKER and FARBER, 1958). The present data would offer further refutation to this concept. However, in those forms of amaurotic idiocy having a slower course and later death than Tay-Sachs disease, our experience has been that there is a notable similarity in brain lipid levels to those found in the older Niemann-Pick children (Groups 'C' and 'D'). The white matter has been found to be only slightly deficient,

but depression is seen consistently in the cholesterol fraction. Gray matter glycolipids are normal or mildly augmented, and sphingomyelin levels are normal.

It is of interest to examine the *relationship between the visceral and cerebral abnormalities* in Niemann-Pick disease. The most extraordinary spleen sphingomyelin levels encountered in this hospital were in three children whose cerebral function is so excellent that it precludes any major chemical abnormality of the brain (Table 6). It would thus seem that cerebral handicaps, when they occur, are separate constitutional phenomena from the visceral abnormality. Regarding world-wide incidence, the most common picture would appear to be that of simultaneous brain and visceral disease, but the data regarding divergence of the phenomena have considerable biological importance. We know of no variation in type of pattern having occurred within the same pedigree.

General comments

The description of Niemann-Pick disease, with or without a coordinate neurologic handicap, has a counterpart in certain other genetically-transmitted syndromes. It has been our experience, for example, that many of the children with the usual 'chronic' Gaucher's disease have had great enlargement of the spleen already identified at the age of two to two and a half years; this implies that there had already been many months of slow accumulation of cerebrosides. Thus, their chemical aberration must have been well-expressed at a time when the nervous system was still immature, but their cerebral function remains normal for a lifetime. The neurological handicaps of the 'infantile' Gaucher's disease patient, however, commonly appear at an early age, when the hepatosplenomegaly is still moderate, and the chemical and histologic study of these patients' brains has revealed only irregular abnormalities. Within the whole category of 'Hurler's disease' or 'gargoylism' there are now at least two well-identified genetic types, similar in skeletal abnormalities, but showing considerable divergence in nervous symptoms. The patients with the 'Hunter' type of sex-linked gargoylism, without corneal clouding, may have near-normal intellectual function in adult life (NJÅ, 1945; MILLMAN and WHITTICK, 1952; SMITH *et al.*, 1952).

From the investigative point-of-view, it would seem most potentially fruitful to approach the problem of the surplus or 'storage' phenomenon first, e.g. the gray matter glycolipid increase in Tay-Sachs disease, and the visceral sphingomyelin surfeit in Niemann-Pick disease. The possibility remains, however, that even if the miracle of replacement therapy were to become available to treat the turnover handicap from an early age, a non-associated inherent neurological abnormality might remain unsolved and equally critical. Because a mixed but generically related group of mutations is currently included within the Niemann-Pick classification, one is probably required to uncover more than one type of explanation for the pathogenesis here, even for the visceral abnormality. Recent studies in our laboratory (CROCKER and MAYS, 1961) have tended to discount the theory of sphingomyelin overproduction in Niemann-Pick disease.

SUMMARY

The lipid content of the developing brain of normal infants and children has been re-investigated, with results reported (for gray and white matter) on 16 patients. By the same techniques, cerebral specimens have been studied from six children with Tay-Sachs disease, and the brains of five Niemann-Pick disease patients re-examined.

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Consistent elevation of the gray matter glycolipids has been noted in the Tay-Sachs material, particularly in the aqueous phase fraction. The white matter lipid levels deteriorated in a steadily progressive fashion with advancing age. Conclusions about the brain in Niemann-Pick disease are hindered by the heterogeneity of the clincial material with this diagnosis. A tentative classification of four clinical and chemical groups is suggested. Gray matter sphingomyelin increases are characteristic only for the 'classical' type of infant patient. The other children show much milder chemical abnormalites in the brain, and it is concluded that the neurological symptoms probably arise from a separate hereditarily-transmitted defect. Separation of the neurological and visceral manifestations is also discussed for Gaucher's disease and gargoylism. Analysis of the brain lipids in children with constitutional syndromes of the sort represented by the lipidoses is a highly desirable investigative procedure, but cannot be expected to offer conclusive information about the nature of the whole disorder in all instances.

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QUANTITATIVE ESTIMATION OF PROTEINS IN EXTRACTS OF CEREBRAL TISSUE AFTER SEPARATION BY ELECTROPHORESIS IN STARCH GEL

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IN A previous communication (BAILEY and HEALD, 1961) it was shown that electrophoresis, in a starch gel medium, of cytoplasmic proteins from brain, revealed the presence of numerous separate components. These components differed in number and type, both according to the species and the area of the brain from which they were obtained. To pursue further problems relating to the possible change in type or quantity of such fractions under various conditions, it was considered necessary to be able to determine the relative quantity of each fraction after separation. Attempts to obtain such information from starch gels have been made by several workers (FINE and WASZCZENKO-Z, 1958; VESSELINOVITCH, 1958). The technique adopted has been to render the gel transparent, after straining the proteins, by embedding in agar, and evaluating the curves obtained by densitometric scanning of the transparent gel. However, evaluations of this type proved difficult and unreliable when applied to fractions separated from cerebral extracts since many of the bands were extremely close together. In consequence, an alternative procedure, in which the stained fractions are first eluted, has been developed.

MATERIALS AND METHODS

Potato starch (Batch No. 023348) and all chemicals were obtained from Hopkin and Williams, Ltd., Chadwell Heath, Essex.

Naphthalene Black 10 B was supplied by G. T. Gurr Ltd., London, S.W.6.

Crystalline bovine serum albumin. This was obtained from L. Light and Co. Ltd., Colnbrook, Berkshire.

Sodium Pentobarbitone ('Nembutal'). This was obtained from Abbot Laboratories Ltd., Greenford, Middlesex.

Pentamethylene tetrazole ('Metrazol'). This was obtained as a solid preparation from British Drug Houses Ltd., Poole. It was dissolved in saline to a concentration of 100 mg/ml before use.

Preparation of starch. Samples of partially hydrolysed starch were prepared as described previously (BAILEY and HEALD, 1961; see SMITHIES, 1955).

Preparation of cerebral extracts. These were prepared and concentrated as described by BAILEY and HEALD (1961) without modification.

Preparation of gels, electrophoresis and staining. The methods previously described (BAILEY and HEALD, 1961) were used without modification with the exception of the slicing technique. The cutting guide used was 1 mm deep, enabling four thin slices of gel to be cut. The two outermost slices were discarded and the inner slices were stained for 20 min in naphthalene black (10 B) (1% w/v) dissolved in a mixture of methanol, water and glacial acetic acid (75:25:10 by volume). The excess dye was eluted with methanol–water–glacial acetic acid (50:50:10 by volume). Fourteen protein fractions are separable from extracts from rat brain and the stained bands were therefore numbered 1 to 14 from the

cathodic end of the gel. These sections, viewed in transmitted light, were cut out with a sharp blade and arranged in numerical order on a clean glass plate. Blank sections, equivalent in size to the stained areas, were also cut. The colour was eluted into 0·1 M-aqueous KOH, either directly from the gel or from the sections which had been air dried at 30°–40°. The latter procedure is more suitable with large sections since the volume of eluant can then be kept reasonably low (about 4·0 ml). After eluting by standing at room temperature for 80 min, the solution was removed and the optical density determined at 620 mμ in 1 cm cells.

Treatment of animals with anaesthetics, Metrazol and electroshock. The animals used were adult male Wistar rats. In each experiment littermates were used as experimental animals and controls. Anaesthesia was induced either by the intraperitoneal injection of Nembutal (0·1 ml/100 g body wt) or by subjecting the animals to strong ether vapours. Metrazol (0·1 ml/100 g body wt) was administered intraperitoneally. Electroshock was administered to young rats, three months old, via electrodes clipped over the ears which had been smeared with an electrode jelly. Shocks of 20 mA, at 50 V for a duration of 5 sec were given and the animal sacrificed 30 sec later when in convulsions. Animals were sacrificed by a blow on the neck, the brain was removed, and extracts prepared as described previously.

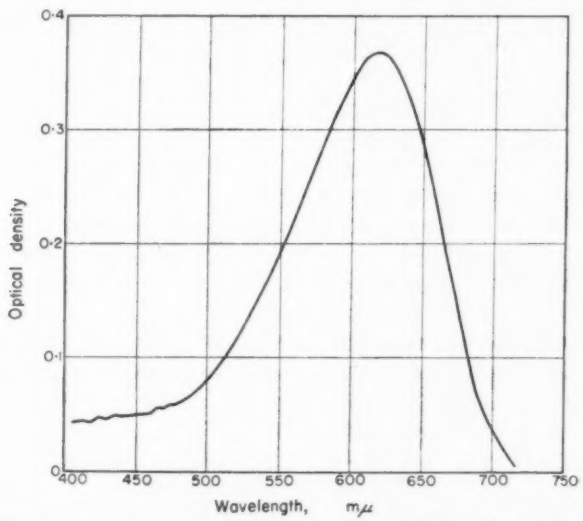

FIG. 1.—The Absorption spectrum of 0·001% (w/v) naphthalene black 10B in 0·1 N-KOH.

RESULTS

Initially attempts were made to estimate the relative percentage of the stained proteins by densitometry of starch gels which had been rendered transparent by the method of FINE and WASZCZENKO-Z (1958). This procedure involved the embedding of a sliced gel in molten agar. After cooling, gels of a high degree of transparency were obtained. The transparency could be further increased by soaking the gel in mixtures of glycerol and water. However when subjected to densitometric measurements in a commercial scanning apparatus, the resolution of the individual components was found to be very poor. Attempts with this technique to obtain quantitative estimates of known amounts of standard bovine serum albumin after electrophoresis, were equally unsatisfactory. Variations of up to 180 per cent were recorded in repeated attempts to assess the relative quantities of albumin present in two standard solutions. This method was abandoned in favour of the elution procedure detailed below.

The samples of dye supplied were found to be uniformly satisfactory in possessing a major component with a well defined absorption maximum at 620 mμ (Fig. 1).

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Ascending chromatography in ethanol–water (1:1 v/v) revealed the presence of five components, two of which were yellow and red respectively. The remaining three were green and blue. All except the blue component were present in very small amounts and clearly did not affect the shape of the absorption curve.

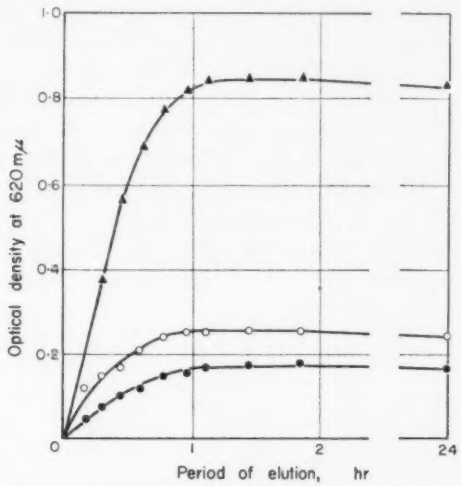

FIG. 2.—The time course of elution of the naphthalene black 10B–protein complex from starch gels.

Different quantities of albumin were separated, stained for 20 min, and the time course of elution by 0·1 N-KOH was determined by optical density readings at 620 mμ. ●—● 25 μg albumin; ○—○ 50 μg albumin; ▲—▲ 150 μg albumin.

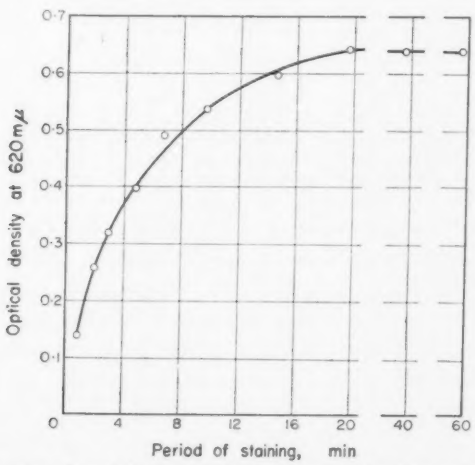

FIG. 3.—The uptake of naphthalene black 10B by albumin after separation in starch gel. 100 μg samples of albumin after electrophoresis were stained with napthalene black 10B (1% w/v) for different times. The coloured complex was eluted for 1·5 hr with 0·1 N-KOH before reading at 620 mμ.

With bovine serum albumin, it was found that, following staining with naphthalene black and removal of the background colour, the dyed protein could be readily eluted by placing the appropriate sections of the stained gel in dilute alkali. Under these

conditions, the elution of the dyed protein was complete in 1–2 hr (Fig. 2), the colour remaining stable for at least 24 hr after elution. With the gels and conditions used the uptake of the dye by the protein was complete in 20 min (Fig. 3). The time period of elution and staining is considered to be a function of the thickness of the gel and is likely to be less for thinner sections.

The proportionality between the intensity of the colour eluted and the concentration of the protein in the gel was examined by first subjecting various dilutions of three separate standards of albumin to electrophoresis in individual starch gels. After staining, the stained areas were cut out, eluted and the colour intensity plotted against the quantity of albumin taken. It was found that the relation between these two obeyed Beers Law up to 150 μg protein (Fig. 4). Amounts above this are far in excess of the quantity present in any single fraction following separation of brain proteins. In each determination recorded in Fig. 4, the 'blank' section of the gel was equivalent in area and volume to that of the section containing the protein.

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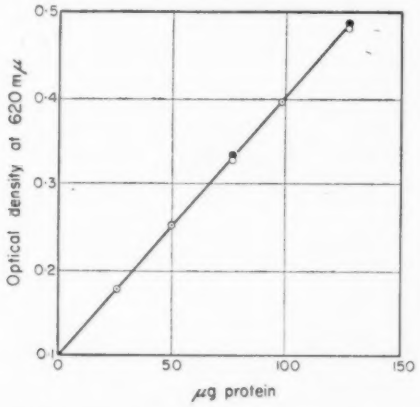

FIG. 4.—The relationships between colour intensity and protein concentration. After electrophoresis the protein samples were stained for 20 min with napthalene black 10B (1% w/v) and the protein-bound dye was eluted with 0·1 N-KOH for 1·5 hr.

The reproducibility of the method was checked both with crystalline bovine serum albumin and by estimating the relative percentages of ten stained components separated from extracts of guinea pig brain. The results of three such comparisons with brain extracts are given in Table 1. Each extract was estimated following electrophoresis in four separate gels, no attempt being made to apply the same quantity of extract to each gel. After separation and staining, the bands were eluted and estimated in the usual way. It will be seen that the individual percentage variation of any given component was usually 1–2 per cent of the total protein and rarely exceeded 5 per cent. This degree of variation is very similar to that found in the denditometric assay of serum proteins separated by electrophoresis on paper (CROOK, HARRIS, HASSAN and WARREN, 1954).

Effects of various stimuli on cerebral proteins. The early experiments of HYDEN (1943, 1947) demonstrated that the protein content of selected areas of the brain were markedly depleted following the application of various stimuli. Although it has since been well established that the cerebral proteins are metabolically active (see RICHTER,

TABLE 1.—THE REPRODUCIBILITY OF INDIVIDUAL DETERMINATIONS OF PROTEIN FRACTIONS SEPARATED FROM EXTRACTS OF GUINEA PIG BRAIN BY ELECTROPHORESIS IN A STARCH GEL

Each extract was assayed on four separate gels, for experimental details see Methods. Values represent % total protein estimated ±S.E.M. of the four separate determinations.

Fraction No.	Extract No.		
	1	2	3
1	1·51 ± 0·22	1·90 ± 0·46	1·51 ± 0·42
2	1·08 ± 0·20	1·17 ± 0·32	0·74 ± 0·31
3	2·79 ± 0·30	4·59 ± 0·40	1·42 ± 0·22
4	6·91 ± 0·40	6·43 ± 0·78	5·25 ± 0·34
5	26·22 ± 1·60	23·05 ± 1·03	24·40 ± 0·72
6	19·92 ± 1·63	16·95 ± 0·85	18·00 ± 0·65
7	26·70 ± 0·60	24·27 ± 3·00	30·37 ± 2·16
8	11·36 ± 1·00	11·62 ± 1·0	13·61 ± 0·88
9	2·05 ± 0·30	3·52 ± 0·43	3·67 ± 0·49
10	1·53 ± 0·50	2·43 ± 0·15	1·57 ± 0·20

1959 for review), relatively little information is available concerning changes in quantity resulting either from different physiological conditions or from applied stimuli. Thus, GORDON and NURNBERGER (1955) using a technique similar to that of HYDEN, showed that the cytoplasmic proteins of the supraoptic neurones increased in quantity during a 1 hr exposure of rats to a cold environment. KIYOTA (1957) found that proteins separated by paper electrophoresis from human epileptogenic tissue, contained increased quantities of the α-globulins. Similar changes were also stated to occur in the cerebral proteins of rats anaesthetised with various barbiturates or with nitrous oxide (INESI, GIANNI, FRANCHI and DESSI, 1957; INESI and GRECO, 1958). Changes, interpreted as indicating a breakdown of cerebral proteins, were found to have occurred in extracts of proteins from cat or dog brain, following the application of electroshock for a few seconds *in vivo* (UNGAR, ASCHIEM, PSYCHOYOS and ROMANO, 1957). It was of interest therefore to examine the cytoplasmic proteins of brain from animals subjected to anaesthetic or convulsive stimuli (Table 2).

Although variations in quantity of certain fractions were detectable in extracts from animals of different litters, particularly fractions 6 and 7, these differences did not occur between litter mates. Fixing the brain by freezing the whole animal in liquid nitrogen revealed no differences between extracts prepared from such brains and those prepared from brains obtained in the normal manner. Examination of eight groups of animals showed that neither anaesthetisation nor convulsive activity resulted in any significant difference in the relative quantities of protein found in the various fractions. It is realised that the method of estimating the fractions obtained by horizontal starch gel electrophoresis is only applicable if there is no electrodecantation. With the system of separation used here there was no evidence of electrodecantation.

DISCUSSION

The procedure described above presents a simple method of determining the relative quantities of the cytoplasmic proteins of brain after separation by electrophoresis in starch gel. The dye chosen, naphthalene black, has been extensively used

TABLE 2.—THE EFFECT OF FREEZING, ANAESTHESIA AND CONVULSIONS UPON THE CYTOPLASMIC PROTEINS OF RAT BRAIN

Adult male rats were used in all experiments. Littermates served as control and experimental animals in each group of determinations. Values are mean percentages of the total protein calculated from four determinations on each group of animals. Two animals were used for each determination. In all instances the variation of the determinations fell within the range given in Table 1. Control animals were untreated.

Animal group	Treatment of animals	Duration of treatment	Fraction numbers													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Frozen in liquid nitrogen	—	3·5	4·1	5·6	5·1	6·8	16·4	10·7	23·0	17·7	5·8	2·2	—	—	—
	Control	—	3·05	4·75	8·4	8·1	9·7	13·5	7·7	22·0	15·7	5·8	2·2	—	—	—
2	Frozen in liquid nitrogen	—	8·6	4·8	4·8	5·2	4·2	26·9	20·9	10·6	5·2	5·3	3·3	—	—	—
	Control	—	8·9	3·2	2·9	4·7	4·9	22·1	28·2	9·4	5·5	3·9	5·3	—	—	—
3	Ether anaesthesia		1·25	2·1	1·45	3·45	4·2	36·6	9·4	6·9	11·7	14·1	5·3	2·7	1·5	2·8
	Control		2·1	1·9	3·7	5·1	7·1	32·0	10·0	6·1	8·5	11·1	4·9	2·2	1·9	3·1
4	Nembutal	2 hr	1·5	1·2	4·4	4·1	17·6	14·3	11·6	10·0	10·27	11·7	5·8	3·9	2·6	1·0
	Control		1·6	1·5	5·2	3·9	16·2	13·7	12·1	11·3	11·2	10·6	5·05	3·55	2·35	1·4
5	Nembutal	2 hr	2·8	2·0	1·6	3·1	4·2	20·6	17·0	8·4	8·2	7·7	11·8	7·15	2·2	3·0
	Control		2·0	3·0	1·9	2·0	2·1	15·8	13·9	12·0	12·1	10·5	12·0	7·62	4·8	2·07
6	Nembutal	2 hr	2·0	1·95	3·7	5·1	7·1	32·0	10·0	6·3	8·5	11·1	4·9	2·2	1·9	3·1
	Control		2·0	4·37	3·7	3·8	5·2	33·0	8·1	6·6	12·0	11·1	3·9	2·7	1·0	2·8
7	Electroshock		5·8	5·8	6·4	7·2	10·8	13·3	8·6	12·6	10·3	13·7	5·3	—	—	—
	Control		5·4	6·0	7·1	7·7	8·4	14·0	8·0	11·8	10·0	14·5	5·7	—	—	—
8	Electroshock		6·7	6·8	5·6	6·0	11·2	11·5	11·2	12·1	10·2	6·4	5·5	6·5	—	—
	Control		6·2	6·8	6·0	6·7	10·3	13·3	9·9	11·3	10·2	6·2	6·0	6·8	—	—
9	Metrazol (convulsions for 1 min)		1·5	1·3	2·7	3·7	14·0	17·9	9·3	7·2	10·9	9·4	10·0	4·7	4·2	3·7
	Control		1·0	1·9	3·5	5·5	15·5	16·7	9·0	9·2	8·3	9·6	8·7	3·8	3·8	3·6
10	Metrazol (convulsions for 2 min)		2·5	1·9	2·5	3·0	6·7	22·2	17·3	7·5	10·7	7·8	6·8	6·3	2·6	2·6
	Control		3·2	2·8	3·1	3·6	7·6	17·6	11·7	7·5	10·3	7·9	6·8	6·7	3·4	4·6
11	Metrazol (convulsions for 2 min)		0·5	0·5	1·5	2·5	6·7	5·7	24·4	11·1	12·5	11·9	10·7	5·9	3·1	3·1
	Control		1·1	0·7	2·1	4·1	5·3	6·5	26·8	11·0	11·75	10·5	9·2	5·1	3·0	2·9

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for the location and estimation of proteins separated by electrophoresis on paper, the high extinction coefficient (GRASSMANN and HANNIG, 1954) making it useful for quantitative estimations. Although the samples used contained several minor components, the smooth absorption curve (Fig. 1) suggested that these were not likely to affect the results when measurements were made at the absorption maximum. It is also recognised that different protein fractions may be stained to different degrees. Such differences are not considered to be of significance when comparisons are being made of the amount of dye taken up by proteins in similar fractions. This is supported by the degree of reproducibility of the method (Table 1). As mentioned above, attempts at quantitative comparisons by densitometric measurement of the starch gels after treatment to render them transparent, were not successful. Combined with the higher resolving power of the starch gel technique the present method would thus appear to offer considerable scope for the detection of abnormalities in cerebral cytoplasmic proteins in, for example, pathological specimens.

Measurable changes in quantity of the cytoplasmic protein fractions could not be detected following applied electroshock, convulsions or anaesthesia, suggesting either that the proteins are metabolically inert or that the techniques of measurement are unable to detect any changes which did occur. Electroshock, and anaesthesia, with or without an accompanying change in body temperature, has been shown to significantly decrease the incorporation of radio-active methionine into the total protein fraction of rat brain (GAITONDE and RICHTER, 1956). However, although cytoplasmic proteins incorporate amino acids to differing extents (PALLADIN and POLYAKOVA, 1959) an examination of the effects of electroshock upon such incorporation into the soluble proteins of rat brain failed to show any changes from normal in each of seven fractions examined (DINGMAN, SPORN and DAVIES, 1959). It is now well established that in brain as in liver the metabolism of amino acids is greatest in particles forming the microsomal fraction (LAJTHA, 1959; CLOUET and RICHTER, 1958), and that the proteins of the cytoplasm are generally less active metabolically in this respect. These conclusions are supported by the evidence presented in the present work. It seems likely that previous reports of changes occurring in cerebral proteins as a result of applied stimuli do in fact take place only in selected areas of the brain and that overall changes are detectable only after extensive breakdown has occurred, for example in perfused cat brain (ABOOD and GEIGER, 1955; GEIGER, 1958). At present however, it is concluded that changes in the metabolism and quantity of the cytoplasmic proteins of brain following altered cerebral activity have not yet been detected.

SUMMARY

1. A method is described for the quantitative comparison of proteins separated from cerebral tissues by electrophoresis in starch gel. The stained proteins are eluted with dilute alkali and the absorption measured.

2. With naphthalene black 10 B as the stain, it was shown that the intensity of the colour is proportional to the concentration, up to 150 μg of protein. The eluted colour is stable for 24 hr.

3. The cytoplasmic proteins of rat brain were estimated following anaesthetisation, electroshock or convulsions induced with Metrazol. No changes were detected in any of 14 fractions separated and estimated.

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INCORPORATION OF [^{14}C]GLYCINE INTO GLUTATHIONE IN RAT CEREBRAL CORTEX SLICES

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WAELSCH *et al.* have published many reports on glutathione (GSH)* metabolism in brain tissue and other tissues. Their recent report with [^{14}C]glutamic acid indicated the rapid turnover of GSH in brain tissue (WAELSCH and RITTENBERG, 1941, 1942; WAELSCH, 1952; BERL and WAELSCH, 1958; LAJTHA, BERL and WAELSCH, 1959). MCILWAIN *et al.* determined the exact content of GSH in brain tissue by means of the glyoxalase method and studied the effects of GSH on the carbohydrate metabolism of brain tissue (MCILWAIN, 1958, 1959; MARTIN and MCILWAIN, 1959). More recently DOUGLOUS and MORTENSEN (1956) demonstrated the synthesis of GSH in the brain by isolating cerebral GSH after intracisternal injection of [2-^{14}C]glycine. Most of these experiments were carried out *in vivo*.

The present work was undertaken to obtain information about GSH metabolism of cerebral cortex slices with particular reference to the effects of some neurotropic drugs.

METHODS

Animal treatment. Male and female rats, weighing about 120 g were used. The animals were killed by decapitation. The cerebral cortex and liver were immediately excised and washed in cold 0·9% NaCl solution. The tissue slices were prepared with a Stadie–Riggs type slicer, and 100–150 mg of cerebral cortex and liver slices were suspended in chilled Warburg flasks containing 1·7 ml of Krebs–Ringer phosphate solution of pH 7·4, 0·2 ml of 3% glucose (or 0·2 ml of 0·3 M-sodium succinate solution) and 0·1 ml (1·0 μC) of [2-^{14}C]glycine (or 0·1 ml (5·0 μC) of [1-^{14}C]-DL-glutamic acid) in a 0·9% NaCl solution. The total volume in the flask was 2·0 ml.

The flasks were incubated at 37° under pure oxygen usually for 2 hr. During the incubation period they were shaken, and readings of oxygen uptake were taken at 10 min intervals. At the end of the incubation period the flasks were removed from the manometer, rapidly chilled in crushed ice, and 15 mg of carrier GSH and 2 ml of 10% TCA were added to the incubation mixtures. The GSH was isolated and analysed as described below.

Isolation of GSH. The contents of each flask and washings with 2 ml of 5% TCA were transferred into a Potter–Elvehjem glass homogenizer and homogenized. The acid-insoluble material was separated by centrifugation, and the residue washed three times with 2 ml of 5% TCA solution. GSH was isolated from the combined supernatant solution as copper mercaptide by the method of WAELSCH and RITTENBERG (1941, 1942). Suspensions of the mercaptide in absolute ethanol were deposited on stainless disks for estimation.

Measurement of radioactivity. The GSH mercaptide obtained as described above was placed on stainless steel disks and dried with infrared radiation. The dried materials were weighed, and their radioactivities were measured by the Lauritsen electroscope. The radioactivities were expressed as divisions per minute per g of GSH mercaptide. 1·0 μC of [^{14}C]amino acid gave 489 divisions/min. The data were corrected for the background and the self absorption.

* The following abbreviations are used in this paper: glutathione, GSH; trichloroacetic acid, TCA; methionine sulphoxide, MSO; 2:4-dinitrophenol, DNP.

Determination of GSH. For the determination of GSH concentration the method of GRUNERT and PHILLIPS (1951) was used after rapid freezing of the tissues with dry ice.

[^{14}C]*Compounds.* [2-^{14}C]Glycine was obtained from the Radiochemical Centre (Amersham, England) and Daiichi Kagaku Inc. (Tokyo, Japan). Specific activities of four batchs of [2-^{14}C]glycine were 65·3 μc/1·0 mg, 49·0 μc/1·0 mg, 46·5 μc/1·0 mg and 13·3 μc/1·0 mg, respectively. [1-^{14}C]-DL-glutamic acid was obtained from the Radiochemical Centre (Amersham, England), the specific activity being 26·5 μc/1·0 mg.

Drugs. Isomytal (sodium isoamylethyl barbiturate) (Nippon Shinyaku Inc., Kyoto, Japan); azacyclonol (Frenquel, 2:4-piperidyl benzhydrol hydrochloride) (Merrell Company, Cincinnati, Ohio, U.S.A.); chlorpromazine (Yoshitomi Inc., Osaka, Japan) and methionine sulphoxide (Nutritional Biochemical Corporation).

RESULTS

GSH concentrations of the brain and liver. GSH concentrations are 3–4 times higher in the liver than in the brain (Table 1).

TABLE 1.—GSH CONTENT OF RAT BRAIN AND LIVER

Tissue	GSH content (μg/g tissue)				Mean
Brain	565 434	503 440	613 483	666 487	524 ± 61
Liver	1844	1917	1770	1750	1820 ± 65

Time course of the incorporation of [2-^{14}C]*glycine and* [1-^{14}C]*glutamic acid into GSH.* The incorporation of [2-^{14}C]glycine into GSH of the rat cerebral cortex slices proceeded at a nearly constant rate at least for 2 hours, but after 2 hours of incubation the incorporation proceeded considerably more slowly (Fig. 1). The incorporation of [1-^{14}C]-DL-glutamic acid into GSH proceeded at a nearly constant rate at least for

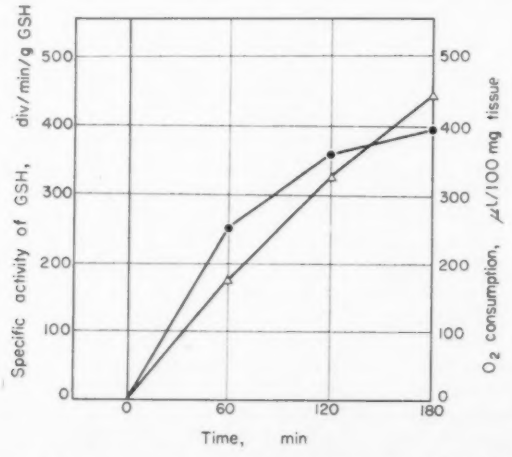

FIG. 1.—Time course of the incorporation of [2-^{14}C]glycine into GSH and the oxygen consumption of rat cerebral cortex slices. Rat cerebral cortex slices (100 mg) were incubated at 37° in Krebs–Ringer phosphate containing 1·0 μc of [2-^{14}C]glycine (sp. act. 49·0 μc/mg) and 0·016 M-glucose. Sp. act. of GSH: div./min/g GSH. Oxygen consumption: μl/g tissue.
● Sp. act. of GSH.
△ Oxygen consumption.

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1 hour, but after 1 hour this incorporation proceeded relatively slowly (Fig. 2). The oxygen uptake proceeded linearly for 3 hours.

Comparison between the cerebral cortex and liver. The respiration was considerably higher in the cerebral cortex slices than in the liver slices (Table 2). The incorporation of [2-14C]glycine into GSH was also significantly higher in the cerebral cortex than in the liver slices.

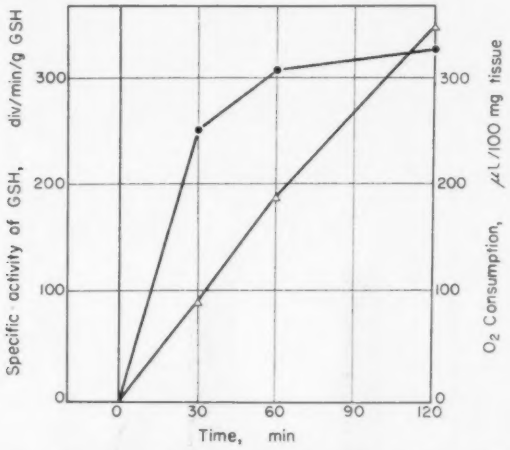

FIG. 2.—Time course of the incorporation of [1-14C]glutamic acid into GSH and the oxygen consumption of rat cerebral cortex slices. Rat cerebral cortex slices (100 mg) were incubated at 37° in Krebs–Ringer phosphate containing 5 μc of [1-14C]-DL-glutamic acid (sp. act. 26·5 μc/mg).
Sp. act. of GSH: divisions per min/g GSH.
Oxygen consumption: μl/g tissue.
● Sp. act. of GSH.
△ Oxygen consumption.

TABLE 2.—THE INCORPORATION OF [2-14C]GLYCINE INTO GSH AND OXYGEN CONSUMPTION OF CEREBRAL CORTEX AND LIVER SLICES

Tissue slices (wt)	Oxygen consumption (μl/2 hr/g tissue)	Sp. act. of GSH (divisions/min/g GSH)
Cerebral cortex (150 mg)	3340	831
Liver (150 mg)	2650	384

Tissue slices were incubated at 37° for 2 hr. For other experimental conditions see legend for Fig. 1 except tissue weight and specific activity of [2-14C]glycine (65·3 μc/mg). The values are means of three experiments.

The effect of DNP. DNP (5×10^{-5} M), which stimulated the respiration of the cerebral cortex slices, showed an inhibitory effect upon the incorporation of [2-14C] glycine into GSH of cerebral cortex slices (Table 3). The respiration and incorporation were equally depressed by 5×10^{-4} M-DNP.

The effects of cysteine and L-glutamic acid. L-Glutamic acid had a considerable inhibitory effect upon the incorporation of [14C]glycine into GSH of the cerebral cortex slices (Fig. 3). Cysteine did not show a significant effect on this incorporation.

TABLE 3.—THE EFFECT OF DINITROPHENOL ON THE INCORPORATION OF [2-^{14}C]GLYCINE INTO THE GSH OF RAT CEREBRAL CORTEX SLICES

Concentration of DNP (M)	Oxygen consumption (μl/2 hr/g tissue)	(%)	Sp. act. of GSH (divisions/min/g GSH)	(%)
Control	3070	100	95	100
1×10^{-3}	—	—	0	0
5×10^{-4}	1120	36	20	22
5×10^{-5}	5510	180	23	24

For the experimental conditions see legend for Fig. 1.
Sp. act. of [2-^{14}C]glycine: 13·3 μc/mg. The values are means of two experiments.

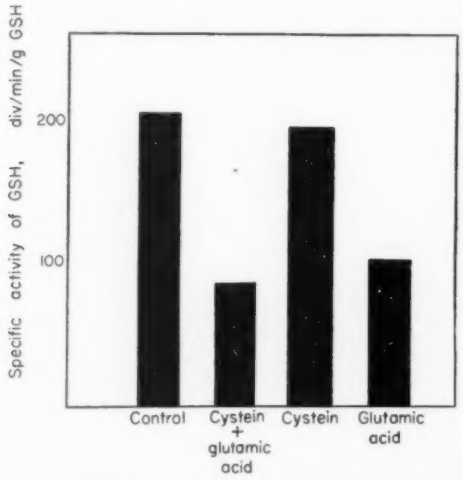

FIG. 3.—The effects of cysteine and L-glutamic acid on the incorporation of [2-^{14}C]glycine into GSH of rat cerebral cortex slices (sp. act. of [2-^{14}C]glycine: 46·5 μc/mg).
For the experimental conditions see legend for Fig. 1 except the addition of cysteine (8 mM) and glutamic acid (8 mM).

Simultaneous addition of the two amino acids showed a similar effect to the effect of the addition of L-glutamic acid alone.

The effects of methionine sulphoxide. MSO did not cause a significant effect upon the respiration, but showed a significant inhibitory effect upon the incorporation of [^{14}C]glycine into GSH of cerebral cortex slices (Table 4). Addition of glutamine significantly inhibited this incorporation. Simultaneous addition of glutamine and MSO showed a similar inhibitory effect.

The effects of some neurotropic drugs. The respiration and the incorporation of [^{14}C]glycine into GSH in the rat cerebral cortex slices were inhibited progressively with the increase of the concentration of Isomytal from 1×10^{-4} M to 1×10^{-3} M (Table 5). The respiration of the cerebral cortex slices was considerably higher in the presence of succinate than glucose. However, the incorporation of [2-^{14}C]glycine into GSH was reduced in the presence of succinate. In the presence of succinate as a substrate Isomytal (even at 1×10^{-3} M) did not inhibit the respiration but inhibited the incorporation of [^{14}C]glycine into GSH.

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TABLE 4.—THE EFFECT OF METHIONINE SULPHOXIDE AND GLUTAMINE ON THE INCORPORATION OF [2-^{14}C]GLYCINE INTO GSH OF CEREBRAL CORTEX SLICES

Concentration (M)	Oxygen consumption (μl/2 hr/g tissue)	(%)	Sp. act. of GSH (divisions/min/g GSH)	(%)
METHIONINE SULPHOXIDE*				
Control	2930	100	638	100
1×10^{-2}	2590	89	345	54
1×10^{-3}	2700	92	421	66
GLUTAMINE†				
Control	3150	100	144	100
1×10^{-3}	3820	121	105	73
1×10^{-4}	—	—	123	86

Rat cerebral cortex slices [*150 mg or †100 mg] were incubated at 37° for 2 hr in glucose Krebs–Ringer phosphate containing 1·0 μc of [2-^{14}C]glycine (sp. act. *65·3 μc/mg, †13·3 μc/mg) and methionine sulphoxide (or glutamine). The values are means of three experiments.

TABLE 5.—THE EFFECTS OF NEUROTROPIC DRUGS ON THE RESPIRATION AND INCORPORATION OF [2-^{14}C]GLYCINE INTO GSH BY RAT CEREBRAL CORTEX SLICES

Substrates	Drugs	Concentration of drugs (M)	Oxygen uptake (μl/2 hr/g tissue)	(%)	Sp. act. of GSH (divisions/min/g GSH)	(%)
Glucose	Isomytal	0	3390	100	452	100
		1×10^{-3}	860	25	58	13
		5×10^{-4}	1920	57	239	53
		1×10^{-4}	2850	84	253	56
	Azacyclonol	0	3500	100	219	100
		2×10^{-3}	—	—	151	69
		1×10^{-3}	3230	92	154	70
		1×10^{-4}	3290	94	167	76
	Chlorpromazine	0	3250	100	170	100
		5×10^{-4}	2200	68	97	57
		1×10^{-4}	2750	85	181	106
		5×10^{-5}	3250	100	173	102
	Acetylpromazine	0	3390	100	208	100
		5×10^{-4}	2070	60	116	56
		1×10^{-4}	2570	76	168	81
Succinate	Isomytal	0	4600	100	320	100
		1×10^{-3}	4220	92	137	43
		5×10^{-4}	4830	105	266	83
	Azacyclonol	0	4170	100	97	100
		2×10^{-3}	4240	102	76	78
	Chlorpromazine	0	4530	100	115	100
		5×10^{-4}	4520	100	75	65

Experimental conditions were similar to those described in Table 4. Rat cerebral cortex slices: 100 mg. Specific activities of [2-^{14}C]glycine were 46·5 μc/mg with Isomytal and 13·3 μc/mg with the other three drugs. The values are means of three experiments except for Acetylpromazine (means of two experiments).

Azacyclonol inhibited both the respiration and the incorporation of [^{14}C]glycine into GSH with increase of the concentration from 1×10^{-4} to 1×10^{-3} M. The inhibition of [^{14}C]glycine incorporation into GSH was usually greater than that of oxygen consumption. Azacyclonol did not inhibit respiration in the presence of succinate as the substrate, but it inhibited the incorporation of [^{14}C]glycine into GSH.

The respiration and the incorporation of [^{14}C]glycine into GSH of the cerebral cortex slices were strongly inhibited by 5×10^{-4} M chlorpromazine. This drug also did not inhibit respiration in the presence of succinate as the substrate but strongly inhibited the incorporation of [^{14}C]glycine into GSH. Acetylpromazine, a derivative of chlorpromazine, caused similar effects upon respiration and incorporation.

DISCUSSION

The incorporation of [^{14}C]amino acids into GSH *in vitro* has been observed in the liver (JOHNSTON and BLOCH, 1951; KRAHL, 1953) and erythrocytes (ELDER and MORTENSEN, 1956) but not in the brain as far as the authors know. The present results indicate that [2-^{14}C]glycine and [1-^{14}C]glutamic acid were incorporated into the GSH of cerebral cortex slices, and [2-^{14}C]glycine into the GSH of liver slices. The incorporation of [^{14}C]amino acids does not necessarily represent a measure of synthesis of GSH but may only be a measure of turnover. The rates of the incorporation of [^{14}C]glycine into the GSH of the cerebral cortex were significantly higher than those of the liver. DOUGLOUS and MORTENSEN (1956) reported that [^{14}C]glycine was incorporated *in vivo* into the brain and liver GSH and that the half life of the GSH was calculated to be about 70 hours in the brain and about 4 hours in the liver. However, a recent report of LAJTHA *et al.* (1959) indicated that the GSH of the brain had high metabolic activity *in vivo*.

Succinate, a substrate that increases the oxygen consumption of cerebral cortex slices, failed to support the incorporation of [^{14}C]glycine into the GSH of cerebral cortex slices, unlike glucose. In the presence of glucose, slices of guinea pig brain could maintain their level of high energy phosphate compounds, while in the presence of succinate they failed to maintain their levels of phosphocreatine and adenosine triphosphate (KRATZING, 1953). Thus, the incorporation of [^{14}C]glycine into GSH may depend upon the nature of the energy donor compounds. This idea is presumably supported by the finding that the addition of DNP causes a great reduction in the incorporation of [^{14}C]glycine into the GSH of cerebral cortex slices.

The addition of L-glutamic acid, alone or together with cysteine, caused a significant reduction in the incorporation of [^{14}C]glycine into GSH of cerebral cortex slices. It is already known that the addition of L-glutamic acid to glucose Krebs–Ringer phosphate medium causes a decrease of the high energy phosphate compounds of brain slices (MCILWAIN, 1951; MCILWAIN *et al.*, 1951). This fact may account for the above findings concerning [^{14}C]glycine incorporation. The inhibition of the incorporation of [^{14}C]glycine into GSH by glutamic acid may also be explained by a dilution of GSH by net synthesis. ELDER and MORTENSEN (1956) showed that the simultaneous addition of cysteine and glutamic acid stimulated the incorporation of [^{14}C] glycine into GSH of erythrocytes. Their observations are not in agreement with the present findings but this may be due to the difference in tissue preparations.

It is known that MSO is an inhibitor of glutamine synthesis (BOREK *et al.*, 1946). RABINOVITZ *et al.* (1956, 1959) reported that the incorporation of [^{14}C]amino acid

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into the proteins of *Ehrlich* ascites tumour and liver was inhibited by MSO, and this inhibition was reversed by the addition of glutamine. The present results indicate the significant inhibition by MSO of the incorporation of [^{14}C]glycine into GSH of cerebral cortex slices. This finding is presumably related to the findings of BOREK *et al.* and RABINOVITZ *et al.* The addition of glutamine also inhibited this incorporation and could not reverse the inhibition by MSO. This observation may be due to a decrease of the high energy phosphate compound of cerebral cortex slices, caused by the addition of glutamine to the glucose Krebs–Ringer phosphate medium, as does the addition of L-glutamic acid (MCILWAIN *et al.*, 1951).

The present results showed that Isomytal, azacyclonol, and chlorpromazine inhibited the respiration and the incorporation of [^{14}C]glycine into GSH of cerebral cortex slices progressively with the increase of the concentration of these drugs. The incorporation was inhibited more than respiration. The observation on the effects of Isomytal and chlorpromazine on the incorporation of [^{14}C]glycine into GSH was similar to that of LINDAN *et al.* (1957) on protein synthesis. These findings showed that the inhibition of the incorporation of [^{14}C]glycine into GSH might be due to the depression of oxidative phosphorylation by these neurotropic drugs, and particularly to the uncoupling of phosphorylation with a low concentration. BRODY and BAIN (1951) and ABOOD (1955) found that the formation of energy-rich phosphate compounds in brain mitochondria was depressed with barbiturate and chlorpromazine. Recently MAGEE *et al.* (1956) showed that the addition of azacyclonol (10^{-3} M) and chlorpromazine (10^{-4}M) to slices of guinea pig brain caused an increase in the labelling of lipid P from inorganic ^{32}P. When the concentration was increased, inhibitory effects were observed. The present findings do not agree with MAGEE's results on phospholipid synthesis. This discrepancy may be due to the difference of the mechanism of the incorporation of [^{14}C]glycine into GSH and of ^{32}P into phospholipid. In the presence of succinate as the substrate the respiration of the cerebral cortex slices was not affected by the addition of these three drugs although the incorporation of [^{14}C]glycine into GSH was inhibited. These observations on the respiration in the presence of succinate were similar to those of LINDAN *et al.* (1957).

SUMMARY

(1) Rat cerebral cortex slices respiring in a glucose Krebs–Ringer phosphate medium incorporated [2-^{14}C]glycine and [1-^{14}C]-DL-glutamic acid into the GSH. The rates of the incorporation of [2-^{14}C]glycine into the GSH of cerebral cortex slices were compared with those of liver slices.

(2) The addition of glutamic acid, glutamine or methionine sulphoxide inhibited the incorporation.

(3) The incorporation was also inhibited by 2:4-DNP in concentrations that do not decrease oxygen consumption.

(4) The addition of succinate as the substrate failed to support the incorporation of [2-^{14}C]glycine into the GSH of cerebral cortex slices.

(5) The respiration and the incorporation of [2-^{14}C]glycine were inhibited progressively with an increase in the concentration of Isomytal, azacyclonol, chlorpromazine, or acetylpromazine. In the presence of succinate as the substrate these drugs did not inhibit the respiration of brain slices.

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IN VITRO INCORPORATION OF [^{14}C]-AMINO ACIDS INTO PROTEINS OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION

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RECENTLY, several workers (GAITONDE and RICHTER, 1956; LAJTHA *et al.*, 1957 and OGATA *et al.*, 1957) studied protein metabolism of brain tissue by using [^{14}C]-amino acids or [^{35}S]methionine. Our group reported the incorporation of [^{14}C]leucine into the microsomal protein by a cell-free preparation of guinea pig brain (SATAKE *et al.*, 1960). However, there were only two reports on protein metabolism of the peripheral nerve with radioactive amino acids up to date: PALLADIN's (1957), studies with [^{35}S]methionine and WAELSCH's (1959) studies *in vivo* with [^{14}C]lysine. The present paper represents findings on the protein metabolism of the rabbit sciatic nerve by using [^{14}C] glycine and [^{14}C]leucine, and the effects of nerve section upon this metabolism.

METHODS

Animal treatment. Rabbits, weighing about 2 kg, were used throughout this experiment. The right sciatic nerve of the rabbit was sectioned or crushed at the greater trochanter of the femur. The operation was carried out as described by JOHNSON *et al.* (1949). After 7, 14, 35, 49, or 91 days the rabbits were killed, and the segment of the nerve distal to the site of the operation was removed. At the same time a similar length of left sciatic nerve was removed to serve as a control. In some experiments the brain was also removed.

Incubation. Each nerve was cleaned of adherent fatty and connective tissue, the epineurium was stripped off, and the nerve was rapidly weighed. The nerve was cut into small lengths (about 1 mm). The cerebral cortex was separated from underlying brain tissue and sliced with a Stadie-Riggs type slicer. The nerve fragments (100 mg) or cerebral cortex slices (100 mg) were transferred to a Warburg flask containing Krebs-Ringer phosphate solution, glucose, and 1·0 μc [2-^{14}C]glycine (or 0·5 μc uniformly labelled [^{14}C]leucine). The total volume of the flask contents was 2·0 ml. The flask was filled with oxygen and incubated in a bath at 37° for 4 hr with shaking. After the incubation, the flask was removed from the manometer and chilled with ice. Two ml 10% TCA solution were added to the flask contents which were homogenized in a Potter-Elvehjem type glass homogenizer. The homogenate was centrifuged, and the protein was purified as described previously (OGATA *et al.*, 1957). In some experiments, the contents of the Warburg flask were centrifuged. The supernatant fluid was taken as "medium fraction" and the remaining cerebral cortex slices or nerve fragments were treated successively with M-NaCl and N-NaOH by homogenization with the solvent in a glass homogenizer; this gave medium, NaCl-soluble, NaOH-soluble, and residual fractions. TCA solution was added to each fraction to a concentration of 5% and the precipitated proteins were purified as described above. The fractionation procedures were performed in a cold room. The protein obtained was transferred to stainless discs and dried under an infrared lamp. The protein was weighed. The estimation of radioactivity was carried out with a Rauritsen electroscope. The specific activity of protein was expressed as div./min/g protein. Correction was made for self-absorption and background. The [2-^{14}C]glycine and uniformly labelled [^{14}C]leucine were obtained from the Radiochemical Centre (Amersham, England). Their specific activities were 46·5 μc/mg and 43·1 μc/mg, respectively; 1.0 μc of [^{14}C]-amino acid gave 489 divisions/min with the Rauritsen electroscope.

RESULTS

Intact nerve

Fig. 1 shows the time course of the incorporation of [2-^{14}C]glycine into the protein of the intact rabbit sciatic nerve and cerebral cortex which had been allowed to respire in the glucose Krebs-Ringer phosphate solution. The specific activity of the protein of both sciatic nerve and cerebral cortex increased linearly as the incubation time was

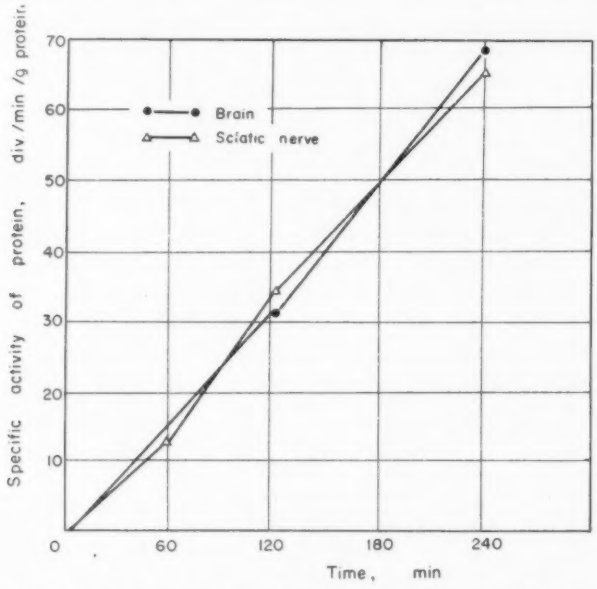

FIG. 1—Time course of incorporation of [^{14}C] glycine into the protein of rabbit sciatic nerve and cerebral cortex.
Sciatic nerve fragments and cerebral cortex slices were incubated at 37° in Krebs-Ringer phosphate containing 1·0 μc of [2-^{14}C]glycine and glucose.

increased up to 4 hr. The specific activity of the protein of the nerve was similar to that of cerebral cortex. Chopping of the nerve into fragments did not result in the diminution of the incorporation of [2-^{14}C]glycine into the protein. However, removal of the epineurium resulted in slight diminution. Since the amount of radio-active carrier-free amino acid present in the medium was very small, the effect of using higher concentrations of amino acid upon the incorporation of [^{14}C]-amino acid into the protein was examined. Addition of more amino acid did not significantly increase the incorporation.

After separate incubation of intact right and left sciatic nerves of the same rabbit for 4 hr in the medium containing glucose and [2-^{14}C]glycine there were no great differences in specific activities of the protein (Table 1).

Sectioned nerve

Nerves degenerating for different periods of time after section were incubated for 4 hr in the medium containing glucose and [2-^{14}C]glycine (or uniformly labelled [^{14}C] leucine). After nerve section the specific activity of the protein increased with each successive period of degeneration (Table 2). At 7 and 14 days after nerve section it

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TABLE 1.—LABELLING OF THE PROTEIN OF THE SCIATIC NERVE OF THE RABBIT

Expt. No.	Specific activity (div./min/g protein)	
	Right nerve	Left nerve
1	57	58
2	59	67

Nerve fragments incubated at 37° in Krebs-Ringer phosphate, pH 7·4, containing 16 mM-glucose and [2-^{14}C]glycine (1·0 μc). Incubation time, 4 hours.

TABLE 2.—EFFECT OF NERVE SECTION ON THE INCORPORATION OF [^{14}C]-AMINO ACIDS INTO THE PROTEIN OF THE RABBIT SCIATIC NERVE

[^{14}C]-amino acid	Time after section (days)	Specific activity of protein (div./min/g protein)	
		Intact control	Sectioned
[2-^{14}C]glycine	7	110	217
	7	89	207
	7	140	318
[U-^{14}C]leucine	7	268	840
[2-^{14}C]glycine	14	96	176
	14	94	189
	14	213	540
[U-^{14}C]leucine	14	668	1650
[2-^{14}C]glycine	35	109	284
	35	48	207
	35	52	533
[U-^{14}C]leucine	49	362	1360
	49	448	875
[2-^{14}C]glycine	91	89	471
	91	131	369

Nerve fragments incubated at 37° in Krebs-Ringer phosphate containing 16 mM-glucose and [2-^{14}C]glycine (1·0 μc) (or uniformly labelled [^{14}C]leucine (0·5 μc)). Incubation time, 4 hours.

reached values 2–3 times greater than that of the control nerve, and at 35, 49, and 91 days it further increased. These results were observed in experiments with [2-^{14}C]glycine or uniformly labelled [^{14}C]leucine.

Of the four subfractions of the protein of the intact sciatic nerve the NaCl-soluble fraction had the highest incorporation of [^{14}C]leucine, followed by the NaOH-soluble fraction, medium fraction, and the residual protein (Table 3). At 14 days after section the specific activities of medium protein, NaCl-soluble protein, and residual protein were significantly greater than that of the controls. The specific activity of NaOH-soluble protein was also greater than that of the control.

Crushed nerve

Table 4 shows the effect of nerve crush on the incorporation of [2-^{14}C] glycine into the protein. At 14 days after crushing the nerve the specific activity of the protein was significantly greater than that of the control nerves.

TABLE 3.—EFFECT OF NERVE SECTION OF THE INCORPORATION OF [^{14}C]LEUCINE INTO THE PROTEIN FRACTIONS OF THE SCIATIC NERVE OF THE RABBIT

Fraction isolated	Mean concentration of protein isolated (mg/100 mg wet tissue)		Specific activity of protein (div./min/g protein)			
			Expt. 1	Expt. 2	Expt. 3	Expt. 4
Medium	Intact control	0·81	84	630	353	381
	Sectioned	1.25	597	930	935	732
M-NaCl	Intact control	0·86	3770	6350	4850	6510
	Sectioned	1·27	7760	12900	12900	9990
N-NaOH	Intact control	0·26	—	—	543	1695
	Sectioned	0·29	—	—	805	1770
Residual	Intact control	17·9	122	237	130	237
	Sectioned	20·1	468	421	589	519

Nerve fragments incubated as described in Table 2. 14 days after nerve section.

TABLE 4.—EFFECT OF NERVE CRUSH ON THE INCORPORATION OF [2-^{14}C]GLYCINE INTO THE PROTEIN OF THE SCIATIC NERVE OF THE RABBIT

[^{14}C]-amino acid	Time after nerve crush (days)	Specific activity of protein (div./min/g protein)	
		Intact control	Crushed
[2-^{14}C]glycine	14	151	512
	14	84	385
	14	179	576

Nerve fragments incubated as described in Table 1. 14 days after nerve crush.

DISCUSSION

There are few reports on the protein metabolism of peripheral nerve. PALLADIN *et al.* (1957) have reported *in vivo* incorporation of [^{35}S]methionine into the protein of the cat sciatic nerve. The present experiments *in vitro* showed that the incorporation of [2-^{14}C]glycine into the protein of the rabbit sciatic nerve was about the same as that of the rabbit cerebral cortex. This observation did not agree with the findings of PALLADIN *et al.* The reason for the discrepancy between these results was not clarified.

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When the proteins of nerve were fractionated into medium, NaCl-soluble, NaOH-soluble, and residual protein, the NaCl-soluble protein had the highest incorporation of [^{14}C]leucine and the residual protein the lowest. This finding was essentially similar to that of PALLADIN *et al.* However PALLADIN *et al.* did not report on the medium fraction. The NaCl-soluble protein contained the nucleoprotein.

WAELSCH (1959) pointed out the possibility that the protein of the frog sciatic nerve might be formed in three ways: (1) protein synthesis in the axon; (2) synthesis in the Schwann cells or glia; (3) synthesis in the corresponding neuronal cell with flow of the axoplasma from the cell body along the axons. Furthermore, his experiments indicated that the last possibility was the most important. However, the present experiments showed that [^{14}C]-amino acids were incorporated into the protein of the sciatic nerve fragments of the rabbit *in vitro*. Therefore the three possibilities of WAELSCH should be considered together.

Sectioned nerve

ROSSITER (1955) summarized many of the chemical changes that take place in the degenerating distal segment of a sectioned peripheral nerve. Particularly, it is noted that ROSSITER *et al.* (1958) studied the incorporation of [^{14}C] precursors and [^{32}P] into the lipids of the intact and sectioned peripheral nerves. However, there are few reports on the protein metabolism of the nerve undergoing Wallerian degeneration. Since the myelin sheath is made up of lipid and protein, the authors carried out the present experiments on the protein metabolism of the sectioned nerves. The findings showed that the incorporation of [^{14}C]-amino acids into the protein of the sciatic nerve increased significantly at 7, 14, 35, 49, and 91 days after nerve section. ROSSITER *et al.* (1958) reported increased labelling of the myelin lipids in degenerating nerve and suggested that this may represent an increase in the capability of the nerve to remyelinate. The present findings may represent a similar phenomenon. In this respect, it is noteworthy that when fragments of rabbit sciatic nerve regenerating after crush were incubated with [2-^{14}C]glycine, there was an increase in the specific activity of the protein. GEREN (1954) reported that the myelin formation was carried out by Schwann cells. Therefore, the present observation may be related to the population of Schwann cells. When the effect of nerve section on the incorporation of [^{14}C]leucine into four subfractions of protein was studied, the specific activities of NaCl-soluble, medium, and residual proteins were significantly greater than those of the controls. This observation is very important when it is considered that the NaCl-soluble protein may contain the nucleoprotein and has the highest specific activity. It is noted that HYDEN (1959) reported the incorporation of [^{14}C]orotic acid into the RNA and [^{14}C]lysine into the protein of the regenerating hypoglossus ganglion cell.

SUMMARY

(1) When the rabbit sciatic nerve was incubated in the Krebs-Ringer phosphate solution containing [2-^{14}C]glycine (or uniformly labelled [^{14}C]leucine) and glucose, the nerve protein was actively labelled.

(2) At 7, 14, 35, 49, and 91 days after nerve section, the incorporation of [2-^{14}C] glycine or uniformly labelled [^{14}C]leucine into the protein of the sectioned sciatic nerve was significantly greater than that of the control.

(3) The incorporation of [^{14}C]leucine into the subfractions of the protein of the

rabbit sciatic nerve was observed. The NaCl-soluble protein had the highest incorporation, followed by NaOH-soluble, medium, and the residual protein. At 14 days after nerve section, the incorporation of [^{14}C]leucine into the NaCl-soluble, medium, and residual proteins increased significantly.

(4) At 14 days after nerve crush, the incorporation of [^{14}C]glycine into the protein of the rabbit sciatic nerve increased significantly.

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RELATIONSHIP OF AMMONIA AND ACETYLCHOLINE LEVELS TO BRAIN EXCITABILITY

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WHILE there have been some earlier studies of brain metabolism during and after convulsions, little is yet known about the neurochemical mechanisms which underlie changes in brain excitability. This is especially unfortunate because of the importance of understanding the trigger mechanism of convulsive activity. Few of the earlier reports even consider simultaneously metabolic changes and seizure pattern.

It is clear that accurate measurement of brain excitability is necessary in a neurochemical study concerned with changing excitability. In this paper, the results of experiments attempting to elucidate the chemical background of changing brain excitability are described. As a first step, an accurate determination of brain excitability in animals was performed. Next, measurements were made of the effect of the chronic administration of several kinds of drugs, which are known to influence the clinical epileptic seizure, on brain excitability and brain ammonia and acetylcholine levels. Finally, brain ammonia and acetylcholine levels in the brain during the course of the seizure, and the refractory period of animals for the seizure were studied.

METHODS

Determination of brain excitability

Two measures of brain excitability were adopted, the seizure threshold and the intensity of seizures. The electroshock seizure threshold (EST)* was used to measure the first factor, and the duration of the tonic extension phase of the maximal electroshock siezure (MES) was used to measure the second. EST and MES were respectively determined using a modification of WOODBURY and DAVENPORT'S (1952) apparatus, by which the interelectrode electric current may be changed independently of the resistance of the experimental animal. By means of a corneal electrode (SPIEGEL, 1937), 50-cycle alternating current was applied for 0·2 sec, the duration being controlled by a pedal switch.

The response of animals to electrical stimulation was classified, according to WOODBURY, as follows: (1) no response, (2) furor, (3) subthreshold stunning, (4) minimal seizure, (5) submaximal seizure (which is further divided into submaximal seizure I and submaximal seizure II; submaximal seizure I shows clonic movements involving the head and forelimbs only, and submaximal seizure II exhibits severe clonic movements of the entire body), (6) maximal seizure (consisting of the tonic flexion phase of the hindlegs, TF, the tonic extension phase of the hindlegs, TE, and the clonic phase of the entire body, Cl).

For determination of EST, the stimulus was delivered twice a week, and EST was determined over a period of 2 weeks before administration of the drugs. EST in rats was defined as the threshold for minimal seizure and was measured to within 0·3 mA. Since minimal seizures are not always detectable in mice, EST in this species was defined as the threshold for submaximal seizure I.

* The abbreviations used are: EST, electroshock seizure threshold; MES, maximal electroshock seizure; TF, tonic flexion; TE, tonic extension; Cl, clonic phase; RT_{50}, recovery time for 50 per cent; DFP, di*iso*-propylfluorophosphate; ACTH, adrenocorticotrophic hormone.

Determination of drug effect on excitability

The drugs investigated were anticonvulsants, hypophysis-adrenocortical hormones, tranquilizing agents and ammonium salts, as shown in Table 1. All drugs were injected subcutaneously once a day for a period of two weeks and their effects on EST and on the duration of the TE of MES were studied.

TABLE 1.—DOSAGES OF INJECTED DRUGS IN EXPERIMENTAL GROUPS

Group	Dosage: mg chemical / kg body wt	Number of rats	effective dose for human: mg chemical / kg body wt
Control	Ringer solution 1·0 ml	10	
Phenobarbital	12	5	2–4
Diphenylhydantoin sodium	40	5	4–6
ACTH	5	5	0·5–1·0
Hydrocortisone	10	5	1·0
Chlorpromazine	25	5	1–2
Reserpine	0·15	5	0·01–0·05
NH_4Cl	150	5	
NH_4HCO_3	160	5	

Determination of the refractory period for the seizure

The refractory period for the seizures in the animals was measured by recovery time (RT_{50}), which is defined by WOODBURY as the time required, after initial electroshock seizure, for 50 per cent of the animals to recover to such an extent that a second electroshock seizure can be elicited. Determinations of RT_{50} for MES and EST seizures were made in adult mice of *dd* strain, each weighing 20–24 g.

Determination of brain acetylcholine, ammonia and glutamine levels in situ

Determinations were carried out for the whole brain, excluding the cerebellum and the portions below the midbrain of adult Wistar albino rats and adult mice of *dd* strain. The animals were killed by immersion in liquid nitrogen. The frozen animals were then decapitated and the brains removed. In the experiments on rats one brain was used in each assay but when mice were used, two or three brains were pooled for each determination. The frozen brain was crushed in a chilled stainless steel crusher by hammering the piston of the crusher (STONE, 1938), and was further ground to a fine powder in a chilled mortar. The powdered brain was transferred to a centrifuge tube of known weight containing the appropriate extraction medium. The wet weight of brain tissue was determined.

Extraction and estimation of acetylcholine. In experiments on rats, acetylcholine was extracted with an eserinized saline (TAKAHASHI, 1958). The powdered brain (about 150 mg wet weight) was stirred with 4 ml of eserinized (260 μg eserine salicylate/ml) amphibian Ringer solution at 0°. The wet weight of tissue was determined by differential weighing. The mixture was rapidly brought to pH 3·8–4·0 with 0·5 N-HCl, then boiled for 2 min, allowed to cool and centrifuged for 10 min at 3000 rev/min. The residue was re-extracted with 1 ml eserinized amphibian Ringer solution, the combined supernatant fluids were brought to pH 6·8–7·0 with 0·5 N-NaOH and used for assay. In experiments on mice, acetylcholine was extracted with a 10 per cent trichloracetic acid solution at −5° for 20 min (CROSSLAND, 1951; CROSSLAND, PAPPIUS and ELLIOTT, 1955). The extracted acetylcholine was measured with the frog *rectus abdominis* muscle preparation described by CHANG and GADDUM (1933)

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with the controls suggested by FELDBERG (1945). None of the drugs used affected the bioassay. Results were expressed as μg acetylcholine chloride per gram of fresh brain tissue.

Estimation of free ammonia and glutamine. The powdered brain was stirred with 4 ml of 12 per cent trichloroacetic acid at 0°. After centrifugation, determinations were carried out on the supernatant solution. The ammonia content of the sample was determined by the Conway microdiffusion technique, using a diffusion time of 60 min at 37° and a half-saturated K_2CO_3 solution (RICHTER and DAWSON, 1948). The amount of ammonia-N liberated by hydrolysis of the brain glutamine under these conditions was equivalent to 3·8 per cent of amide-N of glutamine, and a correction was applied to the brain ammonia values on this basis. The glutamine content in the mice brains was determined separately by the method of HARRIS (1943), essentially as described by RICHTER and DAWSON (1948). The ammonia liberated on hydrolysis with 10 per cent trichloroacetic acid at 70° for 75 min was determined. In the experiment using rats, glutamine content was determined by estimating the ammonia liberated on hydrolysis with half-saturated KOH at 37° for 60 min (MESSER, 1955).

RESULTS

Brain excitability

The EST at room temperature of normal adult rats weighing 150–200 g varied between 15 mA and 20 mA, depending upon the age and weight of the animal. EST in adult mice weighing 20–24 g was found to be 7·3 $\pm$ 0·11 mA (mean value for 10 mice $\pm$ S.E.). MES was induced by currents of 140 mA in rats and 50 mA in mice. MES in rats consisted of an initial tonic flexion lasting 2·7 $\pm$ 0·10 sec (mean value $\pm$ S.E. for 60 rats), followed by tonic extension for 7·8 $\pm$ 0·28 sec, and ending in generalized clonic movments for 5·7 $\pm$ 0·19 sec.

Effects of drugs on excitability

Figs. 1 and 2 show the effects of drug administration on the EST and on the duration of the tonic extension phase of MES.

It is evident from an examination of these figures that EST was elevated in the groups given phenobarbital, diphenylhydantoin-sodium, ACTH and ammonium salts, and lowered in those given chlorpromazine, reserpine and hydrocortisone. The duration of the tonic extension phase of MES was prolonged in the hydrocortisone group and shortened in the other groups. These data indicate that every drug elevating the EST decreased the intensity of MES at the same time. But the drugs (except hydrocortisone) which depressed EST also decreased the intensity of MES. Indeed reserpines, which caused the most obvious depression of EST, had the largest depressant effect on MES.

Effects of drugs on ammonia, glutamine and acetylcholine contents of rat brain

(Results of these determinations are shown in Table 2).

Ammonia content of the brain. The mean value of 0·24 μmole/g obtained for the ammonia content of intact rats in the resting state was almost the same as the value for adult rats reported by RICHTER and DAWSON (1948). As is seen, brain ammonia increased in the hydrocortisone and NH_4HCO_3 groups and decreased in the chlorpromazine group; no significant change occurred in the five other groups. No definite correlation appears when these changes of brain ammonia are compared with changes in EST and MES shown in Figs. 1 and 2. For example, in the elevated EST groups both the increased ammonia group (NH_4HCO_3 group) and the unchanged ammonia groups (diphenylhydantoin-sodium and ACTH groups) occur. Also, the increased ammonia group (hydrocortisone group) is in the lowered EST group. Ammonia

content decreased in the chlorpromazine group, which reduced the duration of MES, but showed no significant change in the reserpine group, which reduced the duration of MES more than the chlorpromazine group. From these observations, it might be concluded that brain ammonia content *in situ* does not correlate with changed brain excitability shown by either EST or MES.

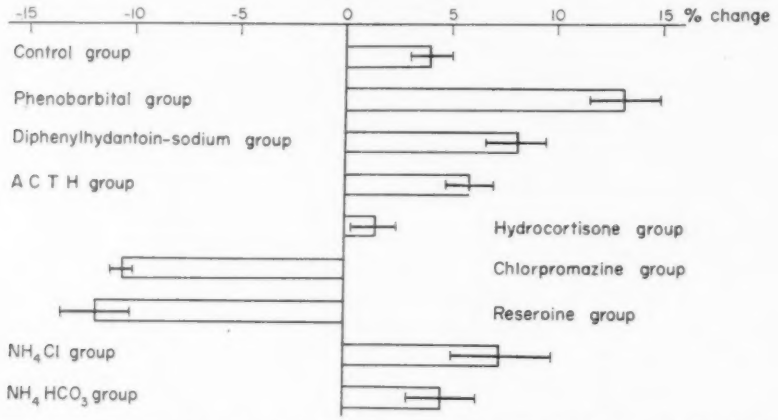

FIG. 1.—Effects of 2 weeks of drug administration on the electroshock seizure threshold of rats. Ordinates give change per cent in EST referred to control value at zero time before treatment. Bars represent standard error of the mean for each group. EST measurements were made 24 hr after the final injection.

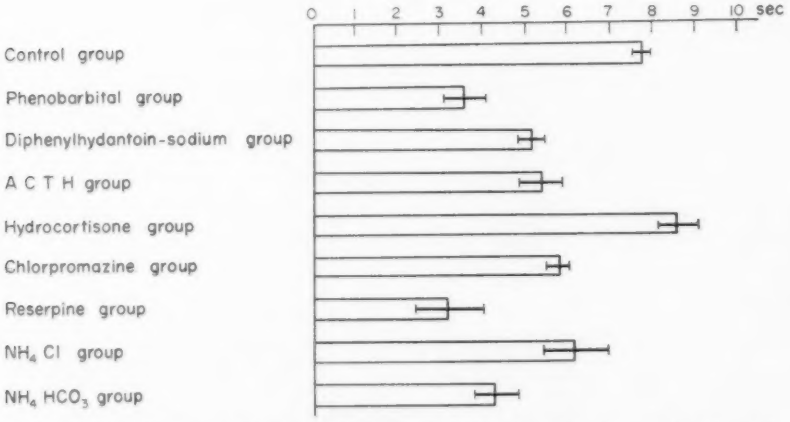

FIG. 2.—Effects of 2 weeks of drug administration on the duration of tonic extension phase of maximal electroshock seizures in rats. Ordinates give the duration of tonic extension of MES. Bars represent S.E. of the mean for each group. MES was elicited 24 hr after the final injection on the 14th day.

Glutamine content of the brain. The glutamine content decreased only in the phenobarbital group, and increased in the diphenylhydantoin, ACTH, chlorpromazine, and NH_4Cl groups. It did not correlate with change in either EST or MES. The ratio of glutamine to ammonia was calculated according to the view that the dynamic equilibrium reaction: glutamic acid + ammonia $\rightleftharpoons$ glutamine participated in functional activity of the brain (VRBA, 1956; KOLOUŠEK, HORÁK and JIRÁČEK, 1959). This ratio

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was higher in the chlorpromazine group and lower in the phenobarbital group. However, it was not found to parallel the change in either EST or MES of all groups.

Acetylcholine content of the brain. The acetylcholine level of the normal adult rat brain, 2·73 ± 0·29 μg/g, (mean ± S.D.) was samewhat lower than the value of 3·1 ± 0·35 μg/g found in the adult rat brain by CROSSLAND, PAPPIUS and ELLIOTT (1955); this may be due to the different method of extraction used in the present experiment.

TABLE 2.—BRAIN AMMONIA, GLUTAMINE AND ACETYLCHOLINE CONTENT OF RATS FOLLOWING 2 WEEKS OF DRUG ADMINISTRATION

Group	Ammonia $\frac{\mu moles}{g\ brain\ wt.}$ ± S.D.	Glutamine $\frac{\mu moles}{g\ brain\ wt.}$ ± S.D.	Acetylcholine $\frac{\mu grams}{g\ brain\ wt.}$ ± S.D.
Intact control	0·24 ± 0·08 (9)	4·78 ± 0·50 (15)	2·73 ± 0·29 (11)
Phenobarbital	0·24 ± 0·12 (5)	4·17 ± 0·56 (5)*	2·90 ± 0·60 (5)
Diphenylhydantoin sodium	0·28 ± 0·12 (5)	5·76 ± 0·64 (5)†	2·75 ± 0·19 (4)
ACTH	0·26 ± 0·10 (5)	5·40 ± 0·42 (5)†	3·07 ± 0·51 (9)
Hydrocortisone	0·32 ± 0·06 (5)*	5·13 ± 0·40 (4)	3·28 ± 0·14 (5)†
Chlorpromazine	0·14 ± 0·09 (4)*	6·30 ± 0·57 (4)†	2·97 ± 0·38 (4)
Reserpine	0·23 ± 0·08 (5)	5·18 ± 0·34 (5)	2·32 ± 0·34 (5)*
NH_4Cl	0·22 ± 0·10 (5)	5·30 ± 0·18 (5)†	2·18 ± 0·25 (10)†
NH_4HCO_3	0·44 ± 0·08 (6)†	4·97 ± 0·39 (6)	

Mean values are followed by standard deviations.
( ) number of experiment is shown in parentheses.
* significant difference of experimental value from control value: $P < 0·04$.
† significant difference of experimental value from control value: $P < 0·01$.
Animals were sacrificed in the resting state, 24 hr after the final injection of drugs.

Acetylcholine increased in the hydrocortisone group, decreased in the reserpine group and NH_4Cl groups, and showed no significant change in the other four groups. When these changes are compared with the results shown in Figs. 1 and 2, it is obvious that acetylcholine level did not correlate with change in EST. However, both the acetylcholine level and the duration of tonic extension of MES increased in the hydrocortisone group and decreased in the reserpine group. The chlorpromazine group, as well as the hydrocortisone and reserpine groups, showed a lowered EST, but chlorpromazine did not produce significant change in brain acetylcholine levels. The fact that there were no observable effects of chronic administration of chlorpromazine on brain acetylcholine is in accord with observations reported by ZELENÝ, LINDAUR and KOZÁK (1957). The effect of the chlorpromazine group on the duration of MES lies intermediately between that of the hydrocortisone group and that of the reserpine group. Thus, a correlation between acetylcholine and the duration of tonic extension of MES can be found in the lowered EST groups. There is, therefore, an indication that acetylcholine levels in the brain may be related to seizure intensity.

The acetylcholine, ammonia and glutamine content of the brains of mice during MES and the duration of the refractory period

In these experiments the time course of the MES pattern and the refractory period of adult mice were measured and compared with brain acetylcholine, ammonia and glutamine level at the same time.

The tonic flexion phase, tonic extension phase and clonic phase of MES of adult mice were respectively 1·1 ± 0·04 sec, 10·4 ± 0·18 sec and 5·6 ± 0·18 sec (mean value ± S.E. for 63 mice). The recovery time (RT_{50}) for MES and EST were calculated by LITCHFIELD and WILCOXON's method (1949) and found to be 84 sec with 95 per cent confidence limits of 77·0–94·0 sec and 15·1 min with 95 per cent confidence limits of 10·0–22·6 min, respectively. RT_{50} for threshold seizure of mice was significantly longer than that reported by BROWN (1953). This may be attributed mainly to the difference in definition of the threshold seizure.

The results of determination of brain acetylcholine, ammonia and glutamine are summarized in Table 3. The mean value of 2·40 μg/g wet weight obtained for the brain acetylcholine level of mice in the resting state is the same as the value reported by CROSSLAND and MERRICK (1954). The acetylcholine content fell rapidly at the tonic flexion phase of MES to 67 per cent of the value at the resting state. During the TE and C1 phases of MES the level of acetylcholine showed gradual recovery towards the normal value. It was especially noteworthy that the acetylcholine concentration returned to normal 84 sec after stimulation, which coincides with the recovery time for MES.

Brain ammonia increased steadily following stimulation and reached its maximal level at the end of the clonic phase. It then began to decrease slowly but did not return to the normal level within the recovery time for MES. The normal level was reached about 15 min after stimulation.

These facts seem to support the possibility that acetylcholine content, but not ammonia content, correlates with intensity of MES.

The glutamine concentration did not change significantly during the maximal electroshock seizure. It showed a small increase 15 min after stimulation. This appears to be the result of an increased rate of fixation of ammonia by glutamic acid.

Effects of anticonvulsant drugs and an acetylcholinesterase inhibitor in mice

(Results of these experiments are given in Tables 4 and 5).

Phenobarbital in a dosage of 40 mg/kg body weight was administered subcutaneously to mice, and then 2 hr later, at the peak time of effect, the animals were stimulated by an electric current of 50 mA for 0·2 sec. Although the animals did not show hypnosis, the tonic flexion phase and tonic extension phase did not appear (because of the anticonvulsant action of the drug); only the clonic phase was apparent and it persisted for about 5 sec (Table 4). In the case of diphenylhydantoin-sodium, administered at the rate of 30 mg/kg body weight, the peak time of effect was 3 hr. Di*iso*-propylfluorophosphate (DFP), an irreversible inhibitor of acetylcholinesterase, was injected at the rate of 2·5 mg/kg body weight, and 30 min later the animals were electrically stimulated, since acetylcholinesterase begins to be resynthesized in the nerve cell 30 min after the inhibition induced by DFP (FUKUDA, 1959). In mice given DFP, all phases of MES were exhibited as shown in Table 4.

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TABLE 3.—BRAIN ACETYLCHOLINE, AMMONIA AND GLUTAMINE CONTENT OF MICE AT DIFFERENT TIMES DURING THE COURSE OF MAXIMAL ELECTROSHOCK SEIZURE AND THE REFRACTORY PERIOD

	Resting state	TF	TE			Cl	Hypokinetic state		RT_{50}(MES)					RT_{50} (EST)
Time after stimulation* (sec)	0	1	3	5	12	17	30	60	84	120	150	180	480	900
Acetylcholine μg/g	2·40 ± 0·11 (9)	1·60 ± 0·20 (7)	1·70 ± 0·17 (4)	1·82 ± 0·06 (4)	1·87 ± 0·05 (3)	1·98 ± 0·11 (3)	2·00 (2)	2·24 ± 0·39 (3)	2·42 ± 0·14 (4)	2·61 ± 0·07 (3)	2·69 (2)	2·45 (2)		
Ammonia μmole/g	0·31 ± 0·06 (7)	0·86 (2)		1·17 ± 0·02 (3)		1·35 (2)			0·69 (2)				0·44 (2)	0·38 (2)
Glutamine μmole/g	4·67 ± 0·56 (7)	4·16 (2)		5·35 (2)		4·85 (2)			4·17 (2)				5·60 (2)	5·86 (2)

Mean values are given ± standard deviations.
Number of experiment is given in parentheses.
* Time elapsed after electric stimulation (50 mA for 0·2 sec).

TF: Tonic flexion phase.
TE: Tonic extension phase.
Cl: Clonic phase.
RT_{50}(MES): Recovery time for MES, RT_{50}(EST): Recovery time for threshold seizure.

TABLE 4.—EFFECTS OF ANTICONVULSANTS AND ACETYCHOLINESTERASE INHIBITOR ON THE PATTERN OF MAXIMAL ELECTROSHOCK SEIZURE

Experimental group	Dosage mg chemical / kg body wt.	Number of mice	TF (sec)	TE (sec)	CI (sec)
Intact control		63	1·1 ± 0·04	10·4 ± 0·18	5·6 ± 0·18
Phenobarbital	40	3	0	0	5·0 ± 0
Diphenylhydantoin	30	5	0	0	5·0 ± 0·55
DFP*	2·5	9	1·4 ± 0·10	9·8 ± 0·32	2·7 ± 0·17†

Values are expressed as mean ± standard error.
† Statistically significant changes at the $P = 0{\cdot}01$ level.
* DFP: di*iso*propylfluorophosphate.

TABLE 5.—EFFECTS OF ANTICONVULSANTS AND ACETYLCHOLINESTERASE INHIBITOR ON BRAIN ACETYLCHOLINE, AMMONIA AND GLUTAMINE CONTENT OF MICE AT DIFFERENT TIMES DURING THE COURSE OF MAXIMAL ELECTROSHOCK SEIZURE

	Experimental group	Resting state	Time after electrical stimulation (sec)				
			1	3	5	12	30
μg acetycholine / g brain wt.	Phenobarbital	2·42 ± 0·04 (3)	2·23 ± 0·03 (3) [CI]	2·15 ± 0·44 (4) [CI]	2·04 ± 0·17 (5) [CI]		2·27 (2)
	Diphenyl-hydantoin	2·39 (2)	2·45 (2) [CI]	1·83 (2) [CI]	2·13 (2) [CI]		2·33 (2)
	DFP	3·19 (2)	3·20 (2) [TF]		4·13 (2) [TE]	4·22 (2) [TE]	
μmole ammonia / g brain wt.	Phenobarbital	0·28 (2)	0·64 (2) [CI]	0·76 (2) [CI]	0·72 (2) [CI]		
	Diphenyl-hydantoin	0·21 (2)	0·88 (2) [CI]	0·62 (2) [CI]	0·82 (2) [CI]		
μmole glutamine / g brain wt.	Phenobarbital	5·65 (2)	5·71 (2) [CI]	5·51 (2) [CI]	5·13 (2) [CI]		
	Diphenyl-hydantoin	5·06 (2)	5·32 (2) [CI]	6·33 (2) [CI]	5·21 (2) [CI]		

The number of experiments is shown in parentheses. Figures in square brackets show the phase of MES at that time.

As shown in Table 5, the brain acetylcholine content of mice injected with either phenobarbital or diphenylhydantoin-sodium was almost the same in the resting state as that of untreated mice. However, in treated mice there was no change in the acetylcholine content 1 sec after electrical stimulation whereas in untreated mice it fell rapidly. The acetylcholine content of treated mice showed a slight fall during the clonic phase and then recovered relatively rapidly. These results seem to be related to the disappearance of the tonic component of maximal electroshock seizure in the animals. Mice given DFP showed a distinctly high value for brain acetylcholine content in the resting state. They exhibited the complete maximal seizure pattern although the clonic phase was significantly shorter than the normal period. There was

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no decrease in brain acetylcholine content throughout the maximal seizure but there was an increase during the clonic phase. This increase seems to be due to accumulation of free acetylcholine in the brain by inhibition of acetylcholinesterase. It also appears that failure of the acetylcholine level to change 1 sec after stimulation in mice given phenobarbital and diphenylhydantoin-sodium is probably due to the retarded rate of release of free acetylcholine from bound acetylcholine since phenobarbital and diphenylhydantoin-sodium are not thought to inhibit acetylcholinesterase.

The brain ammonia content of mice treated with anticonvulsants did not increase as much as that of untreated animals after electrical stimulation.

DISCUSSION

A relationship between brain ammonia and brain excitability was suggested by RICHTER and DAWSON (1948) from the facts that acute administration of ammonium salts caused convulsive seizures in animals, and that the brain ammonia content increased rapidly during the preconvulsive and convulsive phases of seizure induced by the injection of picrotoxin and by electrical stimulation. In this paper, it has been shown that chronic administration of ammonium salts decreased brain excitability with regard to both the seizure threshold and seizure intensity. In these experiments it is considered hardly possible that ammonia given by the injection failed to enter the brain because of detoxication in the liver, since brain ammonia increased in the NH_4HCO_3 experimental group and glutamine increased in the NH_4Cl group. It has been demonstrated that ammonia levels in the brain do not follow changes in excitability. From these observations it may be concluded that the increase of ammonia content in the brain does not initiate convulsive activity, but is the metabolic consequence of functional excitation of nerve tissue. This conclusion is supported by the finding that the ammonia content which increased in mice during maximal seizure did not return to the normal level within the recovery time for the maximal seizure. This conclusion is in agreement with the report by SATO (1959) that seizure discharge did not appear in the electroencephalogram of cats given ammonium salts by intravenous or intracarotid injection, and that convulsive seizures did not occur in cats given ammonium salts by cisternal injection.

It has been known for some time that acetylcholine in the brain might be important in the mechanism of convulsive seizures (RICHTER and CROSSLAND, 1949, CROSSLAND, 1953).

In this paper, it has been demonstrated that the acetylcholine level in the brain is related to the intensity of seizure rather than to seizure threshold, and it has been observed that the time taken for the acetylcholine level to revert to normal after fall at maximal seizure corresponds to recovery time for maximal seizure. This observation supports the view that a sufficiently high acetylcholine level may be one of the requirements for convulsive activity, but other factors, besides acetylcholine level are needed to account for the occurrence of convulsions (RICHTER and CROSSLAND, 1949, TORDA, 1953). It has already been mentioned, that ammonia is not thought to act as one of the initiating factors of convulsion. It has been reported recently that the acetylcholine level in mice brains did not change during the anoxic state, although the ammonia content increased rapidly without the occurrence of convulsions (KATO, 1959)

When the relationship between acetylcholine content in the brain and intensity of seizure is considered, it will be recalled that the administration of both anti-convulsants

and an acetylcholinesterase inhibitor prevented a decrease in acetylcholine level upon electrical stimulation, although the separate effects of the two types of drugs on maximal seizure were quite different. These findings lead us to presume that the rate of liberation of free acetylcholine from the bound form is an important factor in the intensity of seizure. Animals treated with acetylcholinesterase inhibitor could give rise to the maximal seizure pattern after electrical stimulation, and the duration of the clonic movement was significantly shorter than that of control animals. Sufficient information is not available to explain this fact completely, but it does seem reasonable to assume that by the accumulation of free acetylcholine at the synapse of those animal brains, inhibition might have occurred.

While the work discussed in this paper has indicated the possibility that seizure intensity may be partly controlled by the brain acetylcholine level, the neurochemical determinants of the seizure threshold—which may be associated with the trigger mechanism of convulsions—remain so far undefined.

SUMMARY

Ammonia levels in the rat brain, determined after freezing the animals in liquid nitrogen did not correlate with changed brain excitability, measured using either electroshock seizure threshold or maximal seizure duration techniques. Ammonia was thought to reduce brain excitability as a whole. Acetylcholine levels in the brain did not correlate with change in the seizure threshold, however it was shown that acetylcholine level was related to the intensity of seizure. In this case the rate of liberating acetylcholine was thought to be the important factor influencing intensity of seizure.

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EXTRACTION OF LIPID AND LIPID CATION FROM FROZEN BRAIN TISSUE*

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IT WAS pointed out by FOLCH (1947, 1952) and MANERY (1952) that the acidic lipids contain 20 per cent of the anionic sites in brain tissue. Usual extracts of the acidic lipids contain univalent cations in the approximate ratio that exists in whole brain tissue, i.e., 5–7 m-equiv of Na for every 10 m-equiv of K. FOLCH, LEES, and SLOANE-STANLEY (1957) showed that this may be an artefact of the extraction procedures used. These authors found that when salts were added to acidic lipids dissolved in a Folch solution (chloroform–methanol, 2:1, v/v) equilibration with the lipid cation occurs in minutes; the lipid cation will then reflect the distribution of salts present in the entire solution. An exception to the usual finding that the cations of lipid fractions reflect that of the tissue from which the fractions were obtained was reported by KIRSCHNER (1958) who found a procedure for obtaining high sodium phospholipid fractions from high potassium red cells; this indicated that the lipids were preferentially associated with sodium *in vivo*. KIRSCHNER's method included the initial dehydration of the tissues with acetone at −20°, followed by solvent extraction and fractionation. During acetone dehydration at low temperatures chloride salts come into solution and about 25 per cent of the lipid cation exchanges with the inorganic cation as measured by radioisotopes (KATZMAN and WILSON, 1959; KATZMAN, unpublished observations). Yet this amount of interchange of cations was incomplete enough so that high sodium phospholipid fractions could be obtained from high potassium red cells and the sodium content of the phospholipid *in vivo* may well be even greater than that found by KIRSCHNER.

In order to reduce this interchange of cations during extraction of lipids, we investigated other methods of tissue treatment at low temperature. Chloroform, and to a lesser extent, hexane, will elute lipid from brain tissues at temperatures of −45° to −55° without prior dehydration and without removing significant amounts of water. The tissue remains frozen, the cations present as aqueous soluble salts are imprisoned in the ice phase, and the interchange of cations is markedly reduced during extraction.

METHODS

Extraction technique. Tissue slices, obtained as rapidly as possible from the brains of decapitated animals, were frozen in *iso*pentane cooled with liquid nitrogen or dry ice. In the present study, free-hand slices 2 mm in average thickness, as determined by micrometer measurement at the end of the procedure, were used. They ranged between 80 and 160 mg in weight. Frozen slices were treated

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with chloroform at temperatures between −40° and −55°. Since chloroform freezes at −63° lower temperatures are not practicable. Temperatures were maintained by bathing the tubes containing tissue and chloroform in appropriate water–ethanol mixtures, kept in a semi-frozen state with dry ice, the entire cryostat being fashioned according to the method of DEITCH and GODMAN (1955). Glass stoppered, graduated Pyrex centrifuge tubes (30 ml) and glass stoppered, Pyrex reaction flasks (50 ml) were used. Frozen brain tissue floats on chloroform. Hence, the upper surface of the tissue is exposed to air. In the cryostat used the temperature of the air above the tissue averaged −25° to −35°. The temperature at the upper surface of the tissue itself was not measured. To insure a more uniform temperature the tissue can be overlayered with hexane which will float on top of the chloroform, mixing very slowly over several days. The addition of up to 20 per cent v/v of hexane does not measurably affect the rate of elution or the material eluted by the chloroform solvent. At the end of the elution process the solvent was removed through a sintered glass filter stick of medium porosity. Portions were dried under an infra-red lamp and a stream of nitrogen to a constant weight and re-suspended in a measured volume of chloroform. The dry weight of the tissue is the sum of the weight of the extracted material plus the weight of the residual tissue determined by drying to constant weight at 70°. The lipids contained in the residual tissues extractable by the methods of FOLCH *et al.* (1951) are defined as 'residual lipids.'

Analytical techniques. Analytical procedures used include water titration (PETERS and JUNGNICKEL, 1955); cholesterol (SPERRY and WEBB, 1950); lipid phosphorus by a modified FISKE–SUBBAROW method; nitrogen by both Nessler and Kjeldahl; lipid galactose (SVENNERHOLM, 1956); ninhydrin (LEA and RHODES, 1953); choline (APPELTON *et al.*, 1953); serine and ethanolamine (AXELROD *et al.*, 1953). The amount of serine and inositol were checked on paper chromatographs of HCl digests of the lipid materials by comparison of spot size of unknown with spot size of adjacent standards. Lipid chromatography was carried out on silicate paper by the method of MARINETTI and STOLZ (1956). Electrolytes were analysed by the procedure outlined by AMES and HASTINGS (1956). Sodium and potassium analyses were performed with internal standard flame photometry on nitric acid digests. Chloride was determined by the method of LOWRY *et al.* (1954). Calcium and magnesium were determined as the sum of the divalent cations. In determining sodium and potassium at such low levels a problem of some concern is that inaccurate results may occur because of contamination of glassware. To avoid this all glassware was rewashed in nitric acid, and control tubes were analysed. Whenever contamination was found in the control tube the entire run was discarded.

Resistivity measurements. Resistivity measurements were made on frozen solutions and tissue with a highly accurate impedance bridge (Electro Scientific Instruments) equipped with both a d.c. and 1000 cycle a.c. voltage source. Although tissues and solutions at room temperature are subject to electrolysis if a d.c. voltage is applied to them, this phenomenon is minimized when the tissue or solutions are frozen, provided the d.c. voltage is applied momentarily. At impedances above 10^6 ohm-cm which are found in frozen material, the d.c. resistance measurement is simpler and more reproducible than the a.c. resistance measurement since it does not have to be corrected for stray capacitance. The resistivity measurements were made as follows: the solution or tissue to be studied was placed between two electrode plates fixed at a known distance. A thermocouple was embedded in the solution or tissue. The unit was wrapped in a plastic film (Saran) and the entire unit was frozen with *iso*pentane–liquid nitrogen. Light external pressure was applied to keep the metal plates in contact with the tissue or solutions as contraction occurs during freezing. Conductivity measurements were taken both during freezing and warming processes. The cell was standardized against KCl solutions of known conductivity. The measurements obtained by use of this system to measure the resistivity of simple solutions at low temperatures are similar to those reported by REY (1960) (Fig. 1).

RESULTS

Resistivity measurements

To prevent postmortem changes in the distribution of cations, the tissue was frozen and kept at a temperature at which the cations are immobilized in the solid state. This temperature may be approximated by the use of resistivity measurements introduced by GREAVES (1954) and developed by REY (1960). REY was interested in the problem from the standpoint of lyophilization of biological materials, wanting, on the one hand, to preserve materials during lyophilization by keeping them in the solid state

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and, on the other hand, to reduce the time of lyophilization by operating at as high a temperature as possible. To determine the highest safe temperature he attempted an analysis of the eutectics of complex mixtures, using both thermal and electrical measurements. In a simple binary system, very sharp thermal and resistance changes occur at eutectic points: for NaCl this is −22·5° and for KCl it is −11·5°. The resistance at a given temperature is less during cooling than during warming owing to

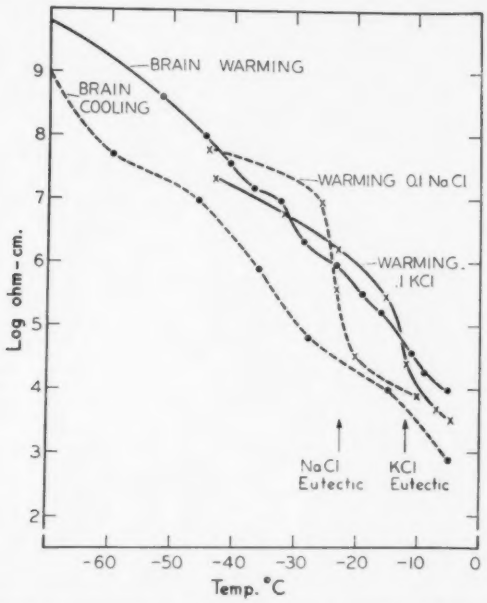

FIG. 1.—Resistivity measurements of whole brain tissue, NaCl and KCl solutions. The measurements were made during the cooling of the tissue and salt solutions and during the subsequent rewarming. The same conductivity cell was used for all materials. Measurements were made with 1000 cycle and d.c. impedance bridges.

supercooling. As mixtures of salt solutions are made, the sharp eutectic border gives way to a broad eutectic zone. According to REY, very complex mixtures have eutectic zones that cannot be predicted from knowledge of the eutectic points of the individual constituents, but must be measured for each complex material. He found that at the temperature at which the resistance of the material becomes greater than 10 MΩ-cm (cooling curve) the material was in the solid state.

Resistivity measurements of brain tissues were made on 5 mm thick sections of brain tissue (Fig. 1). During freezing the brain tissue passes the 10 MΩ-cm point at −46°. During warming this point is recorded at −33°. Just before this latter point is reached, a definite change in the slope of the curve is seen. It would thus appear that the temperature of brain tissue should definitely be kept below −33° and preferably below −46° to ensure a resistance greater than 10 MΩ-cm.

Elution of lipid from frozen brain tissue

Since the purpose of the eluting process is to obtain acidic lipid cation without interchange during extraction, it is desirable to maintain a temperature lower than

−46° if possible. Chloroform freezes at −63° and the solubility of substances in it is reduced as this temperature is approached. Therefore, the amounts of solids eluted in the range of temperatures between −46° and −60° was investigated. Fourteen slices of rat cortex, varying in weight from 82 mg to 124 mg and of approximately equal thickness, were distributed randomly between two Dewar flasks. In one flask the temperature of the freezing mixture was kept between −50° and −60° and in the other,

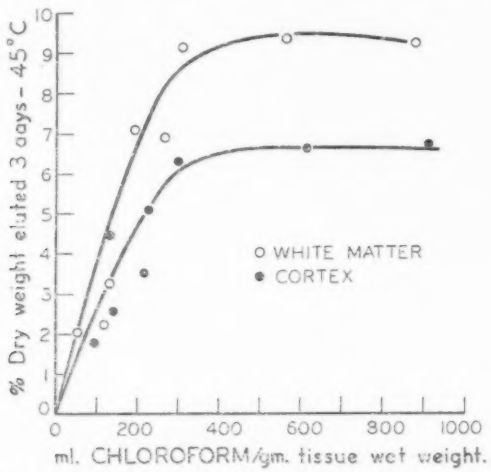

FIG. 2.—Effect of solvent volume on elution of solids at −45°.

in the range of −40° to −50°. Elution was carried out for 3 days. The solids eluted from the tissues kept in the colder Dewar represented 1·7–4·1 per cent of the wet weight with an average of 2·9 per cent, whereas the solids eluted from tissues in the Dewar at the warmer temperature represented 3·1–6·0 per cent of the wet weight with an average of 4·5 per cent. Hence, the amount of material extracted varies with temperature of elution.

Volume of solvent. The amount of solids eluted in 3 days at −45° increases with increasing volume of solvent until a ratio of 200 ml of solvent per gram of tissue is reached (Fig. 2).

Thickness of tissues. The thickness of the tissue does not influence the total amount of matter that is eluted in a 3-day period. Thus, if a tissue slice is crushed after being frozen the total amount of material eluted is not altered. The effect of crushing on the rate of elution was not investigated.

Time course of elution. In these experiments (Fig. 3) the volume of eluting solvent to weight of tissue was maintained at a ratio of over 250, the temperature at −40° to −50°. Under these conditions 90 per cent of the matter that can be removed by chloroform in 9 days is removed within the first 3 days.

On the basis of this information the standard extraction procedure adopted was to use a volume to weight ratio of solvent to tissue of at least 200, to maintain the tissues within the temperature range of −45° to −55° and to continue the elution process for at least 3 days.

Lipid content of tissue. The amount of solid matter eluted is a function of the lipid

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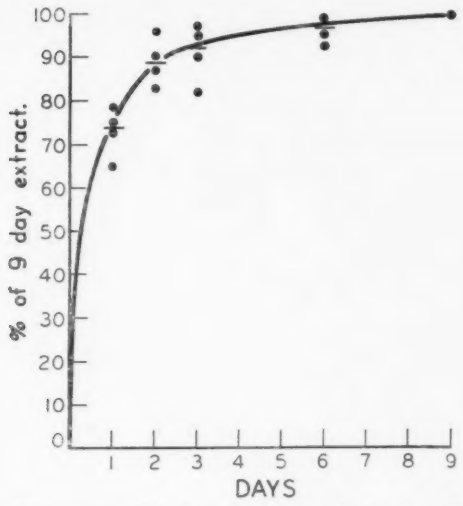

FIG. 3.—Time course of elution of solids from brain tissue slices at −45°.

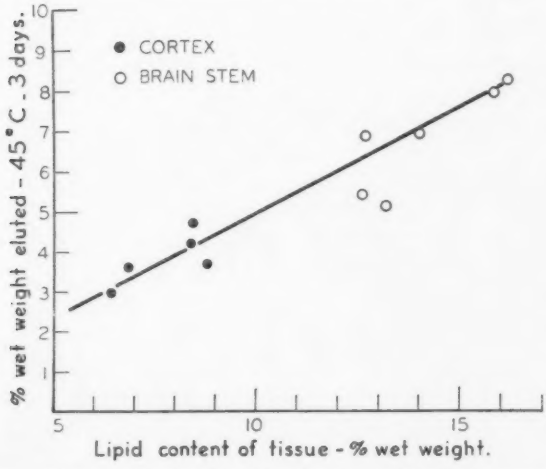

FIG. 4.—Relationship of solids eluted by chloroform at −45° to lipid content of tissue. Slices of brain stem and cortex from rats weighing 80 g–400 g were used.

content of tissue (Fig. 4). Since the proportion of heavily myelinated fibres increases with increasing body weight the lipid content of rat brain increases with the weight of the animal. The weight of the material eluted from these slices is a linear function of the lipid content of the slices. An average of 49 per cent of the tissue lipid was extracted by the chloroform-hexane. This linear relationship may be due to a fairly constant relationship between the sum of the cholesterol and phosphatides and the amount of total lipid in brain tissue.

Elution of water

The amount of water eluted from tissue by chloroform at −40° to −45° was determined at the end of the extraction process by Karl Fischer titration (PETERS and

JUNGNICKEL, 1955). This method is capable of determining 0·2 mg of water in 1 ml of solvent. Chloroform obtained commercially in reagent grade usually contains water at the level of 0·5—1·0 mg per ml of solvent. The solvent may be dehydrated with Zeolite 4A. This dehydration was carried out only for experiments used to determine water eluted since small but significant amounts of solid are added to the solvent by the Zeolite. Titrations were carried out on portions of eluting solvent, these being compared with portions of solvent kept at the same temperature but not in contact with tissue. Both fresh and dehydrated solvent were used. Under these circumstances

TABLE 1.—P:N RATIOS OF DIALYSED AND UNDIALYSED FRACTIONS OF CORTEX

Method	Undialysed			Dialysed		
	P μmole/mg	N μmole/mg	Molar Ratio N:P	P μmole/mg	N μmole/mg	Molar Ratio N:P
FOLCH *et al.* (1951)	0·51	1·39	2·7	0·49	0·83	1·7
MCKIBBIN and TAYLOR (1949)			2·2			1·4
Chloroform-hexane −45°	0·69	0·68	1·0	0·69	0·62	0·9

it was not possible to measure a consistent difference in the water content of the chloroform used to extract a tissue and the chloroform in tubes used as tissue-free controls. Since 25 ml of solvent were used to treat 100 mg of tissue, the amount of tissue water eluted must be less than 5 mg per 100 mg of tissue, or 5 ml per 75 ml of tissue water. Hence, less than 7 per cent of the estimated water content of the tissue is eluted.

Analysis of solids eluted

The solids eluted are chiefly lipids. The evidence for this statement is based in part upon the solubility of the material in chloroform. Protein does not appear to be present in appreciable quantities as the nitrogen present in the extracts is largely accounted for by the nitrogenous lipid bases present. Inorganic salts are not eluted in appreciable quantity by chloroform-hexane at −45° as shown by the chloride content of the extracts which was less than 0·01 μ-equiv. per mg of extract.

Whether or not the extracts contain small non-lipid organic molecules is more difficult to assess. The method used to determine the purity of lipids and their freedom from small organic molecule contamination was to determine the phosphorus to nitrogen ratio before and after dialysis of an aqueous suspension of the lipid (FOLCH *et al.*, 1951, MCKIBBIN and TAYLOR, 1949). In Table 1 the results of the dialysis are compared with those obtained by other methods of lipid extraction. There is a small difference in the molar nitrogen: phosphorus ratio in the dialysed and undialysed extracts. This difference may be due to the presence of small nitrogenous molecules or may represent experimental error. However, the table indicates that if there is contamination of the hexane-chloroform extracts by non-lipids it is not similar in extent to that found with other methods of extraction.

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The lipids extracted by chloroform-hexane at −45° are chiefly the non-sphingosine lipids. In all extracts studied over 90 per cent of the cholesterol was removed from the brain tissue. This extreme solubility of cholesterol at low temperatures was previously found during dehydration of tissues with acetone at low temperatures (KATZMAN and WILSON, 1959). The extracted phospholipid was between 42 and 55 per cent of the total cortex phospholipid and between 38 and 58 per cent of the total brain stem phospholipid. In a few extracts some measurable glycolipid was found, the highest being in a brain stem extract that contained 8·1 per cent of the total brain stem glycolipid. Other extracts contained less glycolipid and in several this was less than 3 per cent of the total brain stem glycolipid. An example of the lipid distribution in extracts of cortex and brain stem of mature 400 g rats is shown in Table 2.

The distribution of the phospholipids in extracts from the cortex and brain stem of mature 400 g rats is given in Table 3. The methods used for analyses of lipid serine and ethanolamine have had an ambiguity of about 15 per cent in our hands but the relative proportions of the extracted phospholipid bases are probably valid. In the extract from cortex by use of hexane-chloroform all of the nitrogen appears to be accounted for by that in choline, serine, and ethanolamine, and, hence, sphingosine appears to be absent. In the extract from brain stem there is a small excess of total nitrogen over the sum of the amounts found in the choline, serine, and ethanolamine, and, hence, some sphingosine is probably present. Within the limits of the analytical method all of the lipid serine was extracted from the cortex by the hexane-chloroform but only half from the brain stem. Serine was also absent on paper chromatograms of hydrolysed samples of the residual lipids of the cortex.

Exchange of cations during extraction

Solubility of chloride salts in chloroform at −45°. Several facts suggest the unlikelihood of appreciable exchange of cations between lipid cation and cation present as an inorganic salt. The chloroform extracts are virtually free of chloride. The aqueous soluble salts appear to remain frozen in the ice phase of the tissue during extraction. Moreover, the solubility of KCl and NaCl in chloroform and hexane at −45° is negligible under the conditions of this experiment. It is so low that the sodium or potassium present in saturated solvent cannot be measured with flame photometry. Approximation was made however by adding radioactive salts of known specific activity to 10 ml of solvent at room temperature. The mixture was rapidly cooled to −45°, shaken, and allowed to stand at −45° for 24 hr to permit any further changes in equilibrium to occur. The solution was removed with a filter stick, the solvent was evaporated, and the residue was treated with 3 ml of nitric acid which was then counted. Since the specific activity of the salt was known, the μ-equiv. of cation could be calculated. The solubility of [^{42}K]Cl in hexane and chloroform was less than 10^{-4} μ-equiv/ml, in acetone intermediate, whereas in Folch solution it exceeded 10^{-1} μ-equiv/ml (Table 4).

Solubility of acidic phospholipid. Whereas the solubility of [^{42}K]Cl in chloroform at −45° is less than 10^{-5} μ-equiv/ml the solubility of K phosphatidyl serine is greater than 25 μ-equiv/ml in chloroform at −45°. Hence, the solubilities differ by a factor of 10^6.

Since the extraction method being described was developed to study the relative proportions of sodium and potassium lipids in brain tissue it is important to know

TABLE 2.—EXAMPLE OF CHLOROFORM-HEXANE TREATMENT OF FROZEN BRAIN TISSUE

Freehand slices, 102–162 mg, of brain tissue obtained from 400 g rats. Slices frozen in *iso*pentane-liquid nitrogen, treated for 4 days in 200 vol. chloroform overlayered with 50 vol. hexane. Temperature of elution, −42° to −50·5°, average −47·4°

Tissue eluted		Solids extracted mg	P (per cent)	Cholesterol (per cent)	Galactose (per cent)	Solids extracted	P-Lipid (P × 23·3)	Cholesterol	Glycolipid (galactose × 4·6)
						g/100 g wet weight			
Cortex									
Combined eluate of 14 slices 1833 mg fresh weight	Chloroform-hexane extract	83	2·58	38·8	0·3	4·53	2·73	1·76	0·1
	Residual lipid*	64	2·83	2·5	6·0	3·49	2·29	0·09	0·98
	Total lipid	147				8·02	5·02	1·85	
Brain stem									
Combined eluate of 14 slices 1524 mg fresh weight	Chloroform-hexane extract	129	2·05	53·3	0·6	8·46	4·04	4·51	0·2
	Residual lipid*	121	1·61	2·2	12 8	7·84	2·99	0·17	4·62
	Total lipid	250				16·30	7·03	4·68	

* Residual lipid refers to the lipid removed by homogenization of the tissue with 2:1 chloroform–methanol following chloroform-hexane extraction. The lipids were washed according to the procedure of FOLCH, LEES and SLOANE-STANLEY (1957).

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TABLE 3.—FURTHER ANALYSES OF LIPID EXTRACTS*

Expressed as moles/g of extract

	P	Total N	Ninhydrin N	Inositol	Choline	Serine	Ethanolamine	Inorganic cations
Cortex								
Chloroform-hexane	0·836	0·84	0·45	0·02	0·33	0·11	0·40	0·15
Residual lipids	0·920	1·5	0·28		0·67	0·01		
Brain stem								
Chloroform-hexane	0·671	0·85	0·49	0·02	0·11	0·08	0·41	0·12
Residual lipids	0·497	1·7	0·22	0·08	0·46	0·09	0·13	

* See Table 2.

whether there is appreciable difference in the solubility of these different lipid salts. Predominantly sodium containing and potassium containing salts of phosphatidyl serine were prepared (FOLCH, LEES, and SLOANE-STANLEY, 1957). Even at the high concentration of salts used (24 μ-equiv/ml), 92 per cent of the sodium phosphatidyl serine and 87 per cent of the potassium phosphatidyl serine were extracted (Table 5). Hence, it appears unlikely that there is sufficient difference between the solubilities of the sodium and potassium phosphatidyl serines to interfere with the method.

TABLE 4.—SOLUBILITY OF [^{42}K]Cl AT $-45°$

10μl [^{42}K]Cl solution added to 10 ml. of solvent, cooled to $-45°$. Precipitation occurred in all solvents except Folch solution. The [^{42}K]Cl added contained 1·9 μ-equiv of K with 351,000 counts/min.

	Total counts/min in solvent	Calculated μ-equiv K/ml solvent
Hexane	180	1×10^{-5}
Chloroform	480	$2{\cdot}6 \times 10^{-5}$
Acetone	36,100	$1{\cdot}4 \times 10^{-3}$
Folch	351,000	$1{\cdot}9 \times 10^{-1}$

TABLE 5.—RECOVERY OF Na AND K PHOSPHATIDYL SERINE IN CHLOROFORM AT $-45°$

(50 mg of mixture of sodium and potassium phosphatidyl serines prepared by the method of FOLCH, LEES and SLOANE-STANLEY (1957) treated with 2·0 ml of chloroform at $-45°$ for 24 hr.)

	Na	K
	μ-equiv	
Mixed salt of phosphatidyl serine	27·9	22·4
Chloroform extraction ($-45°$) of # 1.	25·9	19·4
% recovered	92	87

Measurement of cation exchange with radioisotopes. It might be anticipated that exchange of cation between inorganic salt and acidic phospholipid would be negligible for the following reasons: chloroform extracts are virtually free of chloride; the aqueous soluble salts are frozen in the ice phase of tissue at $-45°$; and solubility of KCl and NaCl in chloroform at $-45°$ is less than that of sodium and potassium phosphatidyl serine by a factor of 10^6. To investigate this supposition [^{42}K]Cl was added to precooled solvent in a frozen droplet of water. A small amount of exchange appeared to occur (Table 6). Somewhat greater exchange occurred if the [^{42}K]Cl was added as a dry salt. The exchange with [^{24}Na]Cl was slightly greater. This exchange was increased appreciably by stirring the mixture with nitrogen. The highest exchange rate occurred when [^{24}Na]Cl was added to a slice of rat cortex, and the mixture stirred with nitrogen. Here, almost 13 per cent of the eluted sodium had exchanged. Probably local heating due to stirring accounts for part of this exchange and, hence, non-stirred solutions have been utilized in further studies. Even with stirring these results are in sharp contrast to

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TABLE 6.—EXCHANGE OF CATIONS IN CHLOROFORM AT −45°

Radioactive salt and either lipid (Folch fraction III containing about 85% phosphatidyl serine) or rat cortex, prefrozen in *iso*pentane-liquid N_2, are treated with 25 ml chloroform at −45° for 48 hr. The specific activity of the eluate is reported as counts/minute μ-equiv cation. The calculated specific activity assumes that complete mixing of the radioactive and non-radioactive cation has occurred

	Materials added as μ-equiv of Na or K			Specific activity of eluate		Ratio
	Radioactive	Lipid	Tissue	Observed	Calculated if complete mixing	Sp. act. obs. / Sp. act. calc.
1 a. [^{42}K]Cl as frozen droplet. Non-stirred	0·08	1·3		38	23,800	0·0016
b. [^{42}K]Cl dry. Non-stirred	0·08	1·3		82	23,800	0·0034
2 a. [^{24}Na]Cl dry. Non-stirred	0·4	2·0		23	4100	0·0056
b. [^{24}Na]Cl dry. Stirred with N_2	0·4	2·0		229	4100	0·056
3. [^{42}K]Cl plus tissue. Mixture homogenized	1·2		1·7	341	8500	0·04
4. [^{24}Na]Cl plus cortex slice stirred with N_2	0·4		0·7	1080	7940	0·13

the findings when similar procedures are carried out with chloroform-methanol solutions even at −40°. Under these circumstances total interchange occurs almost immediately.

Cation present in the chloroform-hexane extracts

The principal cations present in the chloroform-hexane extracts of brain tissue are sodium and potassium. On the average there is 0·14 μ-equiv of sodium plus potassium and less than 0·03 μ-equiv of calcium plus magnesium per mg of lipid extract. The amount of chloride present is less than 0·01 μ-equiv/mg of lipid extract and is at the level to be expected from contamination of the solvent. The amount of total cations

TABLE 7.—MOLAR Na/K RATIOS

	Moles Na/Moles K			
	N	Mean	S.D.	
Rat Brain	36	0·47		(KATZMAN, 1960)
Chloroform-methanol extracts of brain		0·72		(FOLCH *et al.*, 1957)
Cephalin-extracts of rat brain	5	0·69	0·06	(Method of FOLCH, 1942)
Acetone extracts of rat brain −45°	7	0·41	0·07	(KATZMAN, unpublished)
Chloroform-hexane extracts of rat brain −45°	7	1·78	0·29	

present can largely be accounted for by the amount of phosphatidyl serine present in the extracts (Table 3). Some variations in the ratio of sodium:potassium among extracts were found but uniformly the sodium:potassium ratio exceeded 1·0 in all extracts analysed and averaged 1·8.

The ratio of sodium to potassium in these extracts is significantly different from that of whole brain tissue, of chloroform-methanol extracts, of extracts of cephalin obtained by the method of FOLCH (1942), and of acetone extracts from frozen brain tissue at −45° (Table 7). In the latter instance, the tissue was treated with acetone in a manner similar to that used in the chloroform-hexane extractions (KATZMAN and WILSON, 1959). The acetone dissolved water, cholesterol, and inorganic salts from the tissue. The amount of cation removed was of the same order of magnitude as with chloroform-hexane treatment but the molar ratio of sodium to potassium was 0·45 in sharp contrast to the 1·8 ratio found in chloroform-hexane extracts.

DISCUSSION

In the present report a method of extracting the cholesterol and approximately half of the non-sphingosine phospholipids from frozen brain tissue at −40° to −50° has been presented. This method was developed as a means of removing the cation-acidic phospholipid complex intact, i.e., without exchange of cation during the extraction process.

The type of lipids eluted from the frozen tissue may indicate not only the solubility of the lipids in chloroform at −45° but also the nature of the binding of the lipids to structural elements. For example, FINEAN (1957) postulated that the myelin cholesterol present as the free non-esterified molecule is held loosely within the enfoldings of the

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non-polar groups of other lipid molecules. The almost complete elution of cholesterol from white matter by chloroform at −45°, the only lipid moiety to be nearly quantitatively removed, may be consonant with the concept expressed by FINEAN. Moreover, the similar results obtained with grey matter suggest that cholesterol is loosely bound here as well. FINEAN also postulated that phosphatidyl serine present in myelin is attached with its polar groups closely linked to the protein. Since this phosphatide is only partially removed from white matter and almost entirely from grey, these results would be in accord with its state in the former tissue and might indicate that phosphatidyl serine is less firmly held in grey matter.

The mechanism by which chloroform reaches the lipids in a frozen sample of brain tissue is not understood. The sections being extracted are quite thick, being 2 mm in average diameter. Even with allowance for the rather considerable trauma and the holes torn in the tissue during slicing, there must be large volumes of the tissue not accessible to the chloroform on the surface. Yet the amount of lipid extracted from the sliced tissue was as great as from tissue that was crushed and powdered after freezing.

Nor is the explanation to be found in the formation of ice crystals. Even after rapid freezing water kept at temperatures above −130° gradually forms microcrystals as molecular rearrangement occurs. But it is unlikely that the tissue frozen with liquid nitrogen-*iso*pentane and then kept at −40° is sufficiently disrupted by ice crystals to permit the passage of chloroform since such tissues have been shown to remain morphologically intact (FEDER and SIDMAN, 1958).

A likely possibility is that the chloroform moves into the tissue along lipid pathways. Thus the cell membranes which form a closely connected lattice in brain tissue could form such a pathway. Myelin might form additional pathways.

It is important to emphasize that the extracted acidic phospholipids represent a well defined fraction of the total acidic lipids but only a fraction. If it is assumed that there is one anionic site available at tissue pH on each molecule of phosphatidyl serine and each molecule of sulphatide and two anionic sites (there may be three) on each molecule of inositol phosphatide, then, by utilizing the figures of FOLCH and LEBARON (1957) for the content of each lipid in brain tissue, the anionic sites represented by these lipids can be calculated. One kilogram of brain tissue containing half grey and half white matter would contain 13 μ-equiv of anionic sites from phosphatidyl serine (4 grey, 9 white), 8 μ-equiv from sulphatide, and 5 from inositol phosphatides; a total of 26 μ-equiv.

The elution process would remove about 10 μ-equiv of lipid anion with its associated cation—the 4 μ-equiv of phosphatidyl serine in grey, 4–5 μ-equiv of phosphatidyl serine of white matter, 2 μ-equiv of inositol phosphatides.

The high sodium:potassium ratio in the extracted material is of considerable physiological interest. Recent evidence obtained from electron micrographs indicated that the extracellular space in brain tissue is about 4 per cent of the total volume. KATZMAN (1960) calculated that if this extracellular space were filled with an ultrafiltrate of plasma, the sodium:potassium ratio in the remaining tissue would be 0·5, providing that the sodium and potassium were homogeneously distributed. But there is indirect evidence suggesting that glia may be high sodium cells.

KATZMAN (1960) found glial cell tumours to be uniformly high sodium tissues. TERRY and KATZMAN (1960) found the swelling of glial cells in tri-ethyl tin intoxication to be correlated with an increase in tissue sodium. If this thesis were correct, the high

sodium: potassium ratio found in the lipid extracts might represent the elution of high sodium lipid from glial cells as well as the elution of lipid from neurons of high potassium content—the glial lipid predominating.

The high sodium content of the lipid extract might also represent localized inhomogeneity in the distribution of sodium and potassium within individual cells. KIRSCHNER (1958) obtained high sodium phospholipid fractions from high potassium red cells; this indicates inhomogeneity of cations within the red cell. This inhomogeneity of intracellular cationic distribution could also exist in brain tissue. In the case of brain tissue the inhomogeneity would have to be major in extent since the lipid cation represents such a large fraction of the total cation. The extent of the inhomogeneity would be such that it could not be accounted for by the presence of specific sodium carriers such as phosphatidic acid (HOKIN and HOKIN, 1960). To account for the sodium surplus in our extracts phosphatidic acid would have to be present in amounts of 0·04 to 0·06 moles/gram of extracts and in view of the analyses this is unlikely.

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SUMMARY

A method capable of extracting a portion of the cation–acidic phospholipid complex present in brain tissue without interchange of cation during extraction is described. Half of the tissue lipid and 40 per cent of the lipid cation are eluted by soaking slices of frozen brain tissue in chloroform-hexane at $-45°$ to $-55°$ for 3 days. During this treatment the slices are not dehydrated and non-lipid cation remains in the ice phase. The temperature of extraction is based on measurements of the resistivity of brain tissue, a resistivity greater than 10^7 ohm-cm being found below $-46°$. Radioisotope studies indicate that the exchange of lipid and inorganic cation during extraction is less than 13 per cent.

The cation present in these extracts includes sodium and potassium in an average molar ratio of 1·8 although whole brain tissue contains sodium and potassium in a molar ratio of 0·6. The possible significance of the high sodium content of the lipid extracts is discussed.

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EFFECT OF DRUGS ON AMINO ACID LEVELS IN THE RAT BRAIN: HYPOGLYCEMIC AGENTS

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The use of insulin-induced hypoglycemic coma, though no longer as widespread as formerly (Laqueur and La Burt, 1960) is nevertheless still successfully employed in the treatment of certain types of mental illness. The effect of this and of other hypoglycemic agents on the biochemistry of the brain is therefore of practical as well as theoretical interest. Several investigators have studied the influence of insulin on amino acid levels in the brain. Dawson (1950) was the first to observe the fall in glutamic acid in rat brain during insulin hypoglycemia and Cravioto, Massieu and Izquiero (1951) to demonstrate the considerable increase in brain aspartic acid levels that occurs under these conditions. Other workers have since confirmed these findings (Dawson, 1953; Okumura, Otsuki and Nasu, 1959; Jacobson, 1959). It has also been shown (Jacobson, 1959) that the increase in aspartic acid in insulin-treated rats is not due to its liberation from N-acetyl-L-aspartic acid, the presence of which in the brain was first demonstrated by Tallan, Moore and Stein (1956). Other effects on brain amino acids produced by insulin are a decrease in the level of γ-aminobutyric acid (GABA) and of glutamine (Cravioto *et al.*, 1951).

The aim of the present study was to extend these observations. The levels of ten components in the brain, namely glutamic acid (Glu), aspartic acid (Asp), GABA, glutamine (Glu–NH_2), alanine (Ala), taurine (Tau), glycine (Gly), ethanolamine (EtOH–NH_2), phosphoethanolamine (P.eth), and glutathione (GSH), were determined in brains of rats treated with four different hypoglycemic agents: insulin, tolbutamide, hypoglycin A, and the ketoacid derived from hypoglycin A (2-methylene*cyclo*propanepyruvic acid). Attention was directed to the relationship between blood sugar levels and the change in brain amino acid patterns. The role played by N-acetyl-L-aspartic acid in the rise of aspartic acid in insulin-treated rats was also studied.

MATERIALS AND METHODS

Injection of rats. Wistar rats (male, 250 g) were fasted 24 hr and injected intraperitoneally with the material under investigation dissolved in normal saline. The dose level used is reported in the section on results. Insulin (Zinc) was obtained from Novo, tolbutamide from the Upjohn Company, and crystalline hypoglycin A was prepared from the seeds of *Blighia sapida* (De Ropp *et al.*, 1958). 2-Methylene*cyclo*propanepyruvic acid was prepared from natural hypoglycin by the treatment of the amino acid with L-amino acid oxidase. Each treatment was applied to a group of five rats and most experiments were repeated two or more times.

Preparation of brain extracts. Animals were killed by decapitation, their brains removed at once and frozen with powdered dry ice, the operation taking about 40 sec. Brains from each of the five rats receiving a given treatment were pooled and treated either by a modification of the alcohol procedure of Roberts and Frankel (1950) or by the picric acid extraction procedure used by Tallan, Moore, and Stein (1954). For alcohol extraction the pooled brains were homogenized in three volumes of

ice-cold 80% methanol. The resulting homogenate (30 ml) was centrifuged at 20,000 **g** for 15 min. The pH value of 22 ml was adjusted to pH 4·0 and the sample concentrated to 5 ml *in vacuo*. The concentrate was re-centrifuged after standing in ice water for 1 hr. The picric acid extract, after being freed from picric acid, was lyophilized and redissolved to give 4 ml of solution. Recoveries of amino acids obtained by this means are described elsewhere (DE ROPP and SNEDEKER, 1961).

Chromatographic analysis. The method of sequential one-dimensional chromatography employed for separation of the ten components to be estimated has been described elsewhere (DE ROPP and SNEDEKER, 1961).

Blood sugar levels. These were determined by NELSON's (1944) modification of the method of SOMOGYI, the blood samples (heparinized) being collected at the time the rats were decapitated. Blood sugar determinations were carried out by Mr. H. SIEGRIEST.

Estimation of N-*acetyl*-L-*aspartic acid.* Picric acid extracts were prepared from the brains of 10 rats treated with 20 μ/kg of insulin and of 10 comparable rats injected with saline only. Rats in both groups were killed 3 hr after injection when the insulin-treated rats were in coma. The picric acid extracts were lyophilized and redissolved in 3 ml of the aqueous phase of the system *n*-butanol:water: acetic acid (5:4:1). Each of two columns (15 mm internal diameter) was packed to a height of 350 mm with 40 g of acid-washed Celite (diatomaceous earth) which had been thoroughly blended with 20 ml of the aqueous phase of the above system. Dissolved brain extract (2 ml) was blended with 4 g Celite and packed on top of the column. Each column had a hold-back volume of 58 ml. The solvent phase of the system was allowed to flow through the column at the rate of 0·5 ml/min. Four ml cuts were collected. No ninhydrin reacting material was eluted from the column in the first twenty cuts. Two ml from cuts 1–10 and 11–20 were combined and the pooled material was concentrated to dryness *in vacuo* and redissolved in 4 ml of water. To 2 ml was added enough concentrated HCl to give a 6 N solution which was sealed in a tube and heated at 110° for 30 min. The solution was blown down to dryness twice and redissolved in 2 ml of water. Both hydrolysed and unhydrolysed material was chromatographed on Whatman #1 paper (system: *n*-butanol:water:acetic acid 5:4:1) against a standard solution of Asp and N-acetyl-L-aspartic acid prepared by the method of BARKER (1953). Asp was visualized by treatment with ninhydrin according to the method described by DE ROPP and SNEDEKER (1961). Its level was determined by elution of the ninhydrin colour of the area corresponding to Asp. The identity of this conjugated Asp with N-acetyl-L-aspartic was checked by comparing the position of the acidic material in the unhydrolysed fraction with that of an authentic sample of N-acetyl-L-aspartic acid after chromatography in the above system. The position of the compounds was determined by spraying the sheets with a 1% solution of bromcresol green adjusted to pH 8·0.

Test of significance. Sources of experimental error in amino acid estimates were due to (1) variation in response of individual rats (2) variations in extraction procedure, and (3) variations in chromatography. Errors from source (1) were reduced by pooling five brains in each experiment, those from source (2) by repeating each experiment with both the alcohol and picric acid extraction procedures, and those from source (3) by chromatographing every extract four times in the two systems used. Variations in intensity of ninhydrin colour from one run to another proved impossible to eliminate. Every value for the treated group had therefore to be compared with the corresponding value obtained at the same time for the control group. For this reason all data were analysed by the method of paired replicates (SNEDECOR, 1956).

Except in the first experiment, the data have been presented as the ratios of the level of each amino acid in the treated series to the level of the corresponding amino acid in the control series. This value has been calculated and expressed as a percentage $\left(\frac{D_t}{D_c}\right) \times 100$, D_t = optical density of eluate from paper in treated series, D_c of control run at the same time. The test of significance, calculated from the combined differences of the paired values, was only applied where this ratio fell outside the range 90–110 per cent, it being apparent from an overall estimate of variance that changes smaller than ± 10 per cent would be of doubtful significance. The results of these tests of significance have been included only in Table 1 but are given elsewhere in the text. Following accepted usage values of P of 0·001 or less are accepted as highly significant, between 0·01 and 0·001 as significant, and between 0·05 and 0·01 as probably significant.

RESULTS

Levels of amino acids in fasted and insulin-treated rats. In studies of the levels of ten

brain constituents in fasted rats injected with normal saline (average blood sugar level, 80 mg/100 ml) and in fasted rats injected with 100 u insulin per kg (average blood sugar level, 25 mg/100 ml) the rats were killed 3 hr after injection by which time those in the insulin-treated group were in coma (Table 1). The mean values were derived from five separate experiments from a total of up to thirteen chromatographic analyses. They have been expressed in terms of mg/100 g of tissue (wet weight) to indicate the actual amounts of these components present in the brain. GSH, P.eth, and Tau were the only components not affected by the insulin treatment. The amount of free Asp was doubled as was that of EtOH–NH_2. The Glu content decreased by about 30 per cent and that of Glu–NH_2 was halved. Gly and GABA levels were reduced by about 20 per cent and that of Ala by nearly 30 per cent. All these effects proved on analysis to be either significant or highly significant. Findings regarding the levels of Asp, Glu, GABA, and Glu–NH_2 are in agreement with those given by earlier investigators. No figures appear to have been published previously on the effect of insulin on levels of Ala, Gly, and EtOH–NH_2.

Comparative effects of insulin and other hypoglycemic agents. The insulin-treated rats had blood sugar levels of 20 mg/100 ml but rats in the other groups had levels of 40–50 mg per 100 ml (Table 2). Rats in the insulin group were comatose at the time when their brains were removed (3 hr after injection). Those treated with the other substances (Table 2) did not show abnormal symptoms. Amino acid levels in the insulin-treated group show the typical alteration already described. In the other groups, however, the only effect consistently observed was an increase in GABA. It was not very large but was statistically significant ($P < 0{\cdot}001$). The increase in EtOH–NH_2 in the tolbutamide-treated group was of questionable significance. Tolbutamide in 3 lower concentrations (20, 200 and 400 mg/kg) produced increases in GABA but did not significantly affect the levels of the other brain components.

Interdependence of hypoglycemia and amino acid levels. Insulin at four different levels (4, 10, 50, and 100 u/kg) was administered to fasted rats. The three higher dose levels all lowered blood sugar to below 20 mg/100 ml. and in every instance the typical insulin effect on the amino acid pattern was observed with no significant difference in its magnitude between rats treated with 10 u/kg and those receiving 100 u/kg. Those which received 4 u/kg, however, showed a fall in blood sugar level from 80 mg/ml to around 50 mg/100 ml and in these rats no significant changes in the levels of brain amino acids could be detected.

The rate at which the change in the amino acid pattern of insulin-treated rats took place is shown in Table 3. Although the blood sugar level fell to 26 mg/100 ml within an hour after injection, the major changes in amino acid levels did not take place until 3 hr after injection at which time the animals were in a state of shock. The effect of administration of glucose on the levels of brain amino acids was studied in four groups of rats (Table 4). The first received a saline injection only and was killed 3 hr later; the second received saline followed by an injection of 2 g of glucose in saline and was killed 2 hr after this injection; the third group received 20 u/kg of insulin; the fourth received insulin followed by 2 g of glucose per rat, injected intraperitoneally 3 hr after the insulin when the rats were already in a state of shock. The group receiving insulin alone showed the typical insulin-induced change in amino acid pattern. The insulin-treated group which received glucose and was killed 2 hr later showed a rise in blood sugar level to 100 mg/100 ml and significant increases in all those amino acids whose

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TABLE 1.—AVERAGE LEVELS OF AMINO ACIDS IN BRAINS OF FASTED RATS AND RATS IN INSULIN-INDUCED COMA

Treatment	Amino acids (mg/100 g wet weight)									
	GSH	P.eth	Asp	Glu	Glu-NH_2	Gly	GABA	Ala	Tau	EtOH-NH_2
Control	33	44	30	126	65	17	21	5	42	2
Insulin 100 u/kg.	31	43	61	81	30	13	17	3	42	4
$\frac{\text{Treated}}{\text{Control}} \times 100$	94	98	203	64	46	76	81	60	100	200
Significance (*P*)			<0·001	<0·001	<0·01	<0·02	<0·001	<0·001		<0·001

TABLE 2.—FREE AMINO ACID LEVELS OF BRAINS OF RATS TREATED WITH HYPOGLYCEMIC AGENTS, EXPRESSED AS PERCENTAGE OF CONTROLS
Numbers of pairs of estimates in parentheses

Treatment	Amino acids									
	GSH	Asp	Glu	Glu-NH_2	Gly	GABA	Ala	Tau	EtOH-NH_2	P.eth
Insulin 100 u/kg	99 (9)	204 (10)	64 (13)	47 (4)	79 (3)	81 (11)	73 (11)	100 (3)	197 (7)	101 (5)
Tolbutamide 600 mg/kg	98 (10)	86 (10)	90 (18)	89 (8)	84 (4)	116 (12)	110 (12)	98 (8)	200 (6)	—
Hypoglycin A 140 mg/kg	86 (6)	90 (8)	100 (12)	99 (10)	98 (10)	124 (12)	77 (12)	105 (10)	—	—
Keto acid of hypoglycin A 120 mg/kg	99 (6)	93 (8)	99 (12)	93 (4)	—	126 (12)	84 (12)	100 (4)	—	—

levels had been depressed by insulin. The Asp in this group was comparable in level to that in animals receiving saline and glucose only. Brain amino acids in the control group receiving glucose but no insulin also rose in comparison with those of the control group without glucose.

Effect of insulin on acetyl aspartic level. The presence of N-acetyl-L-aspartic acid in the brain, first demonstrated by Tallan, Moore, and Stein (1956) suggested that the increase in Asp observed in the brains of insulin-treated rats might be due to the breakdown of this substance. Acetylaspartic acid was isolated from the extracts of brains

Table 3.—Free amino acid levels in brains of rats at different times after injection of insulin (20 u/kg)

Time (Hr)	Symptoms	Blood Sugar (mg/100 ml)	Amino acids (% of control)							
			Asp	Glu	Glu-NH	Gly	GABA	Ala	Tau	EtOH-NH_2
1	None	26	131	99	104	114	111	97	103	101
2	None	26	135	97	99	100	101	56	88	100
3	Shock	25	208	77	53	88	86	59	80	124
4	Coma	25	221	70	52	—	85	61	110	181
6	Death	—	—	—	—	—	—	—	—	—

Table 4.—Free amino acid levels in brains of rats injected with glucose following saline or insulin

Treatment	Blood sugar mg/100 ml	Amino acid (% of control)					
		Asp	Glu	Glu-NH_2	Gly	GABA	Ala
Saline 3 ml, 0 hr Sacrificed at 3 hr	85	100	100	100	100	100	100
Saline 3 ml, 0 hr Glucose 2 g/rat, 3 hr Sacrificed at 5 hr	150	115	110	139	121	122	120
Insulin 20 u/kg, 0 hr Sacrificed at 5 hr	14	174	72	65	—	91	64
Insulin 20 u/kg, 0 hr Glucose 2 g/rat, 3 hr Sacrificed at 5 hr	100	118	103	140	157	172	210

of fasted and insulinized rats by a partition column as previously described. Hydrolysis and paper chromatography of the cuts showed that all the conjugated Asp was contained in cuts 5–15 and was therefore well separated from ninhydrin reacting components, the first of which appeared in cut 22. Chromatography of the fraction containing the conjugated Asp in butanol:acetic acid:water (5:1:4) revealed an acid spot on spraying with bromcresol green, the Rf of which was identical with that of N-acetyl aspartic acid (0·66). The total amount of conjugated Asp present in the rat brain per 100 g tissue (wet weight) was calculated to be 42 mg in the control fasted rats and 41 mg in rats in insulin coma corresponding to about 55 mg of N-acetyl-L-aspartic acid per 100 g tissue. From this it appears that the increase in Asp in brains of insulinized rats cannot be attributed to the breakdown of N-acetyl-L-aspartic acid. This is in agreement with findings of Jacobson (1959).

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DISCUSSION

BAXTER and ROBERTS (1960) have attributed the effect of insulin on the amino acid levels of the brain to changes in the amounts of citric acid cycle intermediates consequent on hypoglycemia. A lowering in the glucose level of the brain would result presumably in a lowering of pyruvate. Oxalacetic acid would tend to accumulate because, in the absence of sufficient pyruvate, it would not be condensed to citrate. More oxalacetate would thus become available for transamination with Glu and this would result in the production of Asp and α-keto glutarate. The α-keto glutarate would give rise to further oxalacetate which in turn would provide the basis for further Asp formation.

The present work does not provide the detailed observations on enzymic reactions required to check this theory of BAXTER and ROBERTS. It does show, however, that a rise in Asp of 228 μmoles per 100 g wet weight, which is the magnitude of the increase in this component calculated from the values in Table 1, would require the deamination of at least this number of μmoles of Glu. Actually the decrease in Glu observed in this experiment amounted to 306 μmoles per 100 g wet weight, more than enough to provide the necessary additional amino groups for the extra Asp.

The fact that both GABA and Glu–NH_2 levels fall in the brains of rats in insulin coma suggests that the whole metabolic pathway, α-keto glutarate → Glu → GABA → succinic semialdehyde → succinate (the "GABA shunt") is reversed in the direction of α-keto glutarate. The fall in Ala would naturally result from a fall in pyruvate if we assume that this amino acid arises in the brain from the amination of pyruvic acid rather than from the decarboxylation of Asp. The large rise in EtOH–NH_2 is of interest in view of the role played by this compound in the biosynthesis of choline but no explanation can be offered on the basis of the present findings as to why insulin hypoglycemia should produce such a rise.

It would appear correct to attribute the effects of insulin on the amino acid levels in the brain entirely to the lowering of blood sugar produced by this hormone. The degree of lowering appears to be of major importance. A fall from 80 to 40 mg/100 ml such as could be induced by 4 u/kg of insulin was not accompanied by a significant change in the brain amino acid pattern nor did rats treated with this amount of insulin become comatose. Coma was only observed in rats in which the blood sugar level fell to 25 mg/100 ml or lower and in such animals the typical changes in amino acid levels were observed. Administration of glucose returned the rats to consciousness and also restored brain amino acid levels to normal. Coma and brain amino acid levels appear to be correlated.

None of the three oral hypoglycemic agents, tolbutamide, hypoglycin A or its keto acid, lowered blood sugar levels sufficiently to induce coma, nor did they produce the change in the amino acid pattern typical of insulin at coma-inducing levels. The theory may be correct that tolbutamide exerts its effect by stimulating the pancreas to produce additional insulin but this increase is not sufficient to lower blood sugar to such an extent that brain amino acid levels are changed as they are by coma-inducing doses of insulin. The fact that tolbutamide, hypoglycin A, and its keto acid all bring about a significant rise in GABA suggests that these substances all influence the metabolic pathway whereby this component in the brain is either synthesized or degraded. As they do not affect Glu levels, it would seem logical to suppose that they partially block the transformation of GABA to succinic semialdehyde.

SUMMARY

(1) Treatment of fasted rats with insulin brought about a characteristic change in the levels of certain ninhydrin-reacting substances in the brain: a rise in aspartic acid and ethanolamine, a fall in glutamic acid, glutamine, alanine, GABA, and glycine. Glutathione, phosphoethanolamine, and taurine were not affected.

(2) This effect was only observed when sufficient insulin was used to lower blood sugar to about 25 mg/100 ml and induce a state of coma.

(3) The insulin effect on amino acid levels was reversed by administration of glucose.

(4) Levels of N-acetyl-L-aspartic acid in the brains of comatose insulin-treated rats were not significantly lower than those in control rats.

(5) Oral hypoglycemic agents, tolbutamide, hypoglycin A, and the keto acid of hypoglycin A, did not produce any of the changes in amino acid levels characteristic of insulin, probably because they did not reduce blood sugar levels enough to produce coma. Their only significant effect was to raise the level of GABA.

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HETEROGENEITY OF LACTIC AND MALIC DEHYDROGENASE IN CEREBROSPINAL FLUID*

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RESEARCH on enzymic activity in the cerebrospinal fluid (CSF)† has shown that there is an increase in glutamic–oxalacetic transaminase activity in cerebral arteriosclerosis and in subacute sclerosing leucoencephalitis; in some lipidoses the aldolase (ALD) activity is increased (ARONSON, SAIFER, PERLE and VOLK, 1958).

Compared to the serum, the ratio of enzymic activity to total protein concentration is greater for the CSF. Although the lactic dehydrogenase (LDH) and ALD activity of the CSF is about one tenth of that of the serum, the total protein of the CSF is about 1/200 of that of the serum. The amount of LDH varies with the CSF cell count and not with the total protein concentration.

Recent investigations on enzymes have introduced the concept of tissue specificity. As a corollary of this, a heterogeneity of serum enzymes has been shown by chemical, chromatographic, and especially by electrophoretic techniques. WIEME's (1959) method called 'enzymoelectrophoresis', makes it possible to demonstrate the heterogeneity of LDH and malic dehydrogenase (MDH) in human serum. This method was applied in a preliminary study of the heterogeneity of these enzymes in the CSF (LOWENTHAL, VAN SANDE and KARCHER, 1960) and more detailed results are now given.

REAGENTS AND METHOD

Method. WIEME's (1959) method allows one to separate electrophoretically some serum enzymes into different fractions. The enzymic activity of each fraction is estimated by the changes in optical density which occur during the WARBURG (1949) reaction.

Reagents. 1% Difco Noble agar, USA, in 0·05 M-veronal buffer pH 8·4 (8·5 g sodium diethyl barbiturate, 11·5 ml N-HCl, H_2O to 1000 ml). Reference solution, ASD: albumin, siderophilin and dextran (dialysed Macrodex Poviet) each in 1% concentration in distilled water. Stain: 0·5 g amidoschwarz 10 B (Bayer), 5·0 g $HgCl_2$, 5 ml CH_3COOH, H_2O to 100 ml (filter before use).

Four to 5 ml of CSF obtained by lumbar puncture were concentrated by ultra filtration‡ under pressure (10 atm. N_2, room temperature) for 1–1·5 hr. 0·15 mg protein was applied to a glass slide covered with agar gel. The slide was prepared by heating agar in veronal buffer (see under Reagents) for 10 min at 100° and by pouring the warm solution on to the slide (125 mm × 75 mm) about 12 hr before use. Two cuts at right angles to the length of the slide were made in the centre of the agar layer.

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† *Abbreviations used*—Cerebrospinal fluid, CSF; aldolase, ALD; lactic dehydrogenase, LDH; malic dehydrogenase, MDH; standard mixture of albumin, siderophilin and dextran, ASD; diphosphopyridine nucleotide, DPNH; relative mobility, m_r.

‡ The filters and apparatus used were provided by Membranfilter Gesellschaft, Goettingen, Germany.

The longer (10 mm) was for the concentrated CSF and the other (2 mm) for the standard mixture, ASD. Electrophoresis was carried out at 140 v and 20 mA for 25 min. The electrophoretic chamber was kept cool by the evaporation of petroleum ether (Fig. 1).

A second slide, the reagent slide, was prepared containing the substrate and the coenzyme. To study the LDH activity, this slide was covered with an agar gel containing a solution of Na-pyruvate and disphosphopyridinenucleotide (DPNH). To prepare the gel, 2 ml of 2% agar were heated in a water bath at 100° and, after cooling

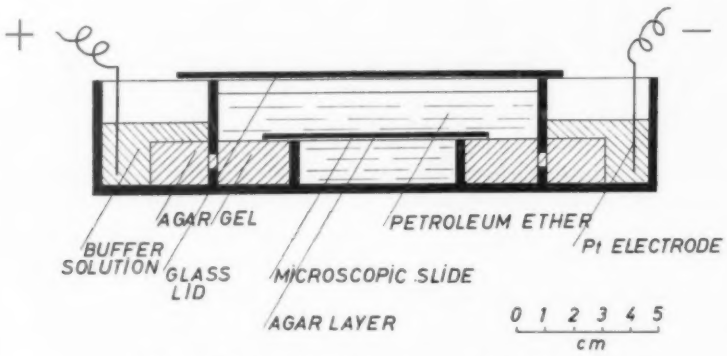

FIG. 1.—Chamber for electrophoresis, after WIEME.

to 50°, 1 ml H_2O, 2 mg DPNH diluted in 1 ml H_2O and 0·1 M–Na-pyruvate were added. This mixture (3 ml) was poured on to the slide, which was held horizontally. For MDH activity the procedure was the same, except that to the heated and cooled agar were added 1 ml of a fresh solution of 0·2% DPNH and 0·3 ml of 0·3 M-oxalacetic acid neutralized with NaOH.

After electrophoresis, the slide with the CSF and ASD proteins was removed from the electrophoretic chamber and placed on the reagent slide. The glass slide used as support for the electrophoresis was then removed. The twinned agar layers were placed in a spectrophotometer* and the optical density was scanned every 0·5 mm at 366 mμ. The first reading, obtained within 3 min was taken as the base line. A second reading was made 30 min after twinning the agar plates when the peaks of enzymic activity are well defined. The curves obtained were computed by planimetry, and the relative concentrations of the different fractions were calculated. The relationship between the enzymic activity of the various fractions and the optical density is an exponential one and these values are only approximate. The points of maximum absorption were marked by making cuts in the agar gel.

Fixing was carried out in a solution of 5% CH_3COOH in 70% C_2H_5OH for 30 min and the reagent plate discarded. The dextran of the standard mixture was marked by a cut in the agar gel, as it does not stain with amidoschwarz. The plate was placed upside down on a strip of filter paper and put into an incubator (37°) for 12 hr. Finally, it was stained with an amidoschwarz solution (see under Reagents) and rinsed several times in 5% CH_3COOH in distilled water. The mobility (m_r) of each enzymic fraction was measured relative to the constituents of the standard mixture.

* Eppendorf colorimeter, modified to permit the study of microscope slides.

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The distance from the standard albumin to dextran was taken as the unit of measurement.

Thus, these methods enable one to express the dehydrogenase activity by two quantitative values: the concentration and the m_r of each of the activity peaks.

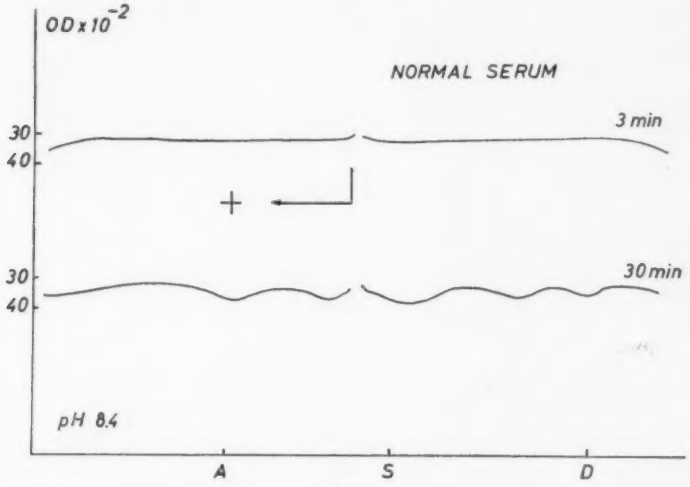

FIG. 2.—Electrophoretic dispersion of the LDH activity zones in human serum.

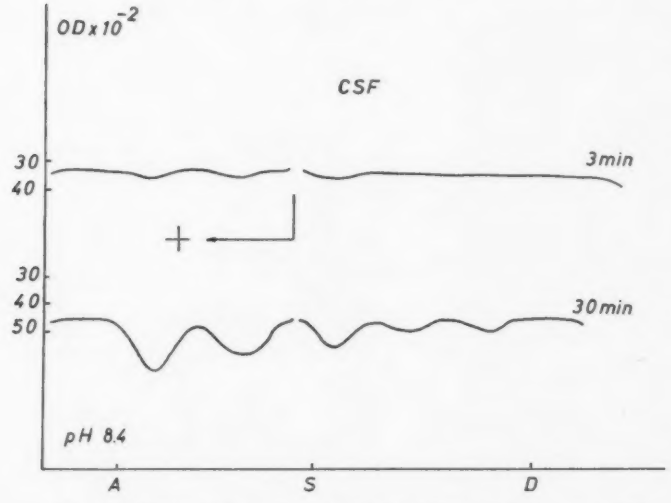

FIG. 3.—Electrophoretic dispersion of the LDH activity zones in CSF.

RESULTS

The electrophoretic patterns of LDH were more pronounced and so more easily read for CSF than for serum (Figs. 2 and 3). Both fluids gave 5 activity peaks. Fraction II in the human serum and fraction I in the CSF were quantitatively the most important (Table 1). The m_r of the various LDH activity peaks were identical for CSF and serum but they did not always correspond to the m_r of the protein fractions

(Table 2). Fraction II coincided exactly with the α_2-globulin; fraction I, however was found between the albumin and α_1-globulin. Fraction III did not quite coincide with the β_1-globulin. Fractions IV and V were not found in all CSFs, and were as a rule less pronounced.

TABLE 1.—RELATIVE CONCENTRATION OF LDH ACTIVITY PEAKS
(expressed in %)

	Number of samples	I	II	III	IV	V
Serum (WIEME)	11	23·2	47·5	18·5	7·5	3·2
Number of samples		11	11	11	8	7
CSF	74	38·5	30·0	24·3	7·1	5·1
Number of samples		74	74	73	42	24

TABLE 2.—RELATIVE MOBILITIES OF THE DIFFERENT LDH ACTIVITY PEAKS

	Number of samples	I	II	III	IV	V
Serum (WIEME)	11	0·932	0·691	0·454	0·218	0·021
CSF	74	0·910	0·677	0·456	0·241	0·073

				Globulins					
		Alb.	α_1	α_2	β_1	β_2	γ_1	γ_2	γ_3
CSF proteins	84	1·005	0·878	0·682	0·472	0·348	0·225	0·142	0·055

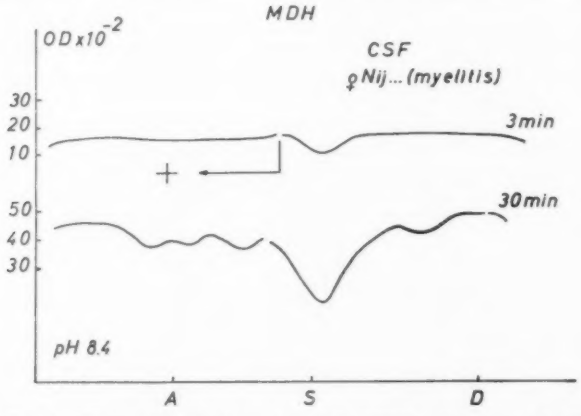

FIG. 4.—Electrophoretic dispersion of the MDH activity zones in CSF.

Similar observations were made for MDH (Fig. 4). The enzymic activity appeared at 6 different locations, of which the 4 fast migrating ones were very constant; the two slower migrating ones were less constant and less pronounced. The first activity peak was found beyond the albumin, between prealbumin and albumin. Fractions II and III had similar mobilities to those of LDH fractions I and II, while MDH fraction IV coincides with LDH fraction III (Table 3). Fraction IV was quantitatively the most important (Table 4). The last 2 fractions were difficult to define.

One wonders how much spontaneous decarboxylation of oxalacetate to pyruvate

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contributed to the appearance of MDH activity peaks II, III and IV, the m_r of which coincide with and partially overlap peaks I, II and III of LDH activity.

Attempts were made to show the presence of sorbitol dehydrogenase, but with the technique used it was not possible to detect this enzyme.

TABLE 3.—RELATIVE CONCENTRATIONS AND RELATIVE MOBILITIES OF MDH ACTIVITY PEAKS (40 samples)

CSF	I	II	III	IV	V	VI
Relative concentration (%)	12·1	12·5	12·9	66·9	9·3	11·2
Number of cases	27	37	39	40	4	3
Relative mobility	1·067	0·905	0·681	0·404	0·255	0·029

TABLE 4.—RELATIVE MOBILITIES OF THE MDH AND LDH ACTIVITY PEAKS IN THE CSF

LDH		0·910	0·677	0·472		0·225	0·073
MDH	1·067	0·905	0·681		0·404	0·255	0·029

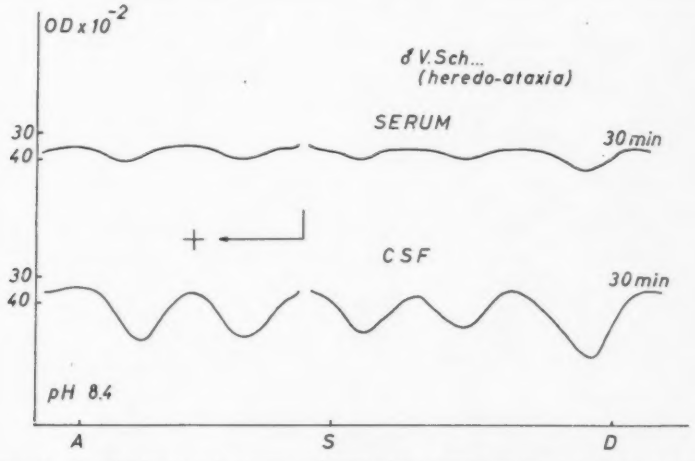

FIG. 5.—Parallel increase in serum and CSF of the LDH activity peak in the γ-region.

In the different neurological diseases under study (arteriosclerosis, epilepsy, various tumours, multiple sclerosis and degenerative diseases) no specific modification of enzymo-electrophoretic pattern was found. However, parallel changes in serum and CSF appeared in some cases (Fig. 5).

CSFs with a high total LDH concentration gave a normal enzymo-electrophoretic pattern.

DISCUSSION

Enzymo-electrophoresis of LDH and MDH of CSF confirmed the heterogeneity of these enzymes as already shown for other biological fluids. The various fractions detected may have different origins and each may be specific for a particular tissue. No definite conclusions are possible for the central nervous system. Perhaps by applying this technique to enzymes in extracts of nervous tissues more definite information might be obtained.

So far, the quantitative differences between LDH from serum and CSF cannot be attributed either to tissue specificity or to selective filtration.

Enzymo-electrophoresis enables one to gather more information about the region extending from albumin to the β-globulins, where most enzymic activities are located. Thus new information is available about the α region

Quantitative estimates of relative concentrations raised certain problems. All the enzymic activity peaks are not always present in the CSF. The arithmetical mean can be calculated either by using the total number of samples, assuming the fractions not detected to be present at a level of 0 per cent, or, by using only those values for fractions which are actually detected. The second method was used for calculations shown in Tables 1 and 3 which explains why the totals of the relative concentrations are greater than 100 per cent. By the first method of calculation the LDH activity peaks for fraction IV and V are 5 per cent and 2·5 per cent respectively; the MDH peaks for fractions I, V and VI become 8·2 per cent, 0·8 per cent and 0·7 per cent respectively.

The problem of the best method for the calculation of the mean remains unsolved for electrophoretic fractions of proteins.

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SUMMARY

Enzymo-electrophoresis has been applied to the CSF. The heterogeneity of LDH and MDH has been demonstrated. Some of the activity peaks are probably due to tissue specificity, although this hypothesis remains unproved.

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THE METABOLISM OF METHIONINE DERIVATIVES CONTAINING ^{35}S IN THE RAT BRAIN *IN VIVO*

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METHIONINE metabolism *in vivo*, and its uptake into different organs, has received attention by a number of authors (GAITONDE and RICHTER, 1955, 1956) and the metabolic activity of this substance has been demonstrated (NIKLAS *et al.*, 1956, 1958; CLOUET and RICHTER, 1959). No attention has been paid, however, to methionine sulphoxide and methionine sulphone, the first and the second oxidation stages of methionine. The destribution of [^{35}S]methionine sulphoximine in the rat has been investigated by ROTH *et al.* (1952) who in a later paper (ROTH *et al.*, 1953) suggested that the cause of the toxic effect of methionine sulphoximine could be the incorporation of this substance in place of methionine into the proteins. In connexion with our studies of the effect of methionine and methionine sulphoximine on epileptic fits in experimental animals (LODIN and KOLOUŠEK, 1956, 1958; KOLOUŠEK and LODIN, 1959) we set ourselves the task of ascertaining whether or not methionine sulphoximine, and the sulphoxide and sulphone are incorporated into proteins.

METHOD

Adult male white rats of 250–280 g were used. The [^{35}S]-DL-methionine used was of Soviet origin and its specific activity was 800 mc/g. [^{35}S]-DL-Methionine sulphoxide and its normer and [^{35}S]-DL-methionine sulphoximine and its normer were, in essence, prepared according to BENTLEY, MCDERMOTT and WHITEHEAD (1951). [^{35}S]-DL-Methionine sulphone and its normer were prepared from [^{35}S]-DL-methionine by oxidation with performic acid and purification of the products by cation exchange.

Methionine sulphoxide, methionine sulphone and methionine sulphoximine were injected intraperitoneally (400 mg/kg body weight), the substances containing ^{35}S being mixed with their normers so that the activity of the injected substances was about 1 mc/kg body weight. The dose of 400 mg/kg body weight was chosen because it corresponds to the paroxysmal dose of methionine sulphoximine which provokes in the rat a series of epileptic fits, the so-called *status epilepticus*, 5–6 hr after i.p. application. Although the incorporation of [^{35}S]-methionine into brain has been studied before by other authors, we also applied [^{35}S]-DL-methionine to the experimental rats (1 mc/kg body weight, i.p.) for comparison with the above mentioned substances. All the applied preparations were chromatographically pure. The relatively low specific activity of the applied substances and the 24 hr duration of the experiment exclude any possibility of artefacts due to radioactive disintegration.

After treatment, the experimental animals were kept in metabolic cages, according to COMMAR, receiving water ad libitum and the urine was collected in calibrated test-tubes. After 2, 6 and 24 hr the animals were killed by decapitation, the heads falling into liquid nitrogen. For each time-interval and for each substance 12 rats were used. The results are given as mean values. The frozen brain was taken from each head and ground to powder under liquid nitrogen. Samples of approximately 1 g in weight were further homogenized in glass homogenization test-tubes with 10 ml of 20% trichloroacetic acid at a low temperature. From these homogenates three fractions were obtained: (1) proteinous, (2) non-proteinous or soluble in trichloroacetic acid and (3) lipidic by the following procedure. The proteins precipitated from the homogenate by 20% trichloroacetic acid were quantitatively filtered and washed with equal volumes of 5% trichloroacetic acid. The filtrate, the non-proteinous fraction,

was evaporated to dryness. The precipitated proteins were dried and extracted for 8 hr in small extractors according to SOXHLET using a chloroform–methanol mixture (2:1). This gave the lipidic fraction which was evaporated to dryness. The fractions were mineralized in Kjeldahl flasks in a sand bath. The sulphur was oxidized to SO_4 and converted to benzidine sulphate which was quantitatively filtered off. The method used was essentially that of GAITONDE and RICHTER (1955). The precipitate, a disc 16 mm in diameter, was measured by a G-M tube with a mica window of thickness 3·5 mg/cm². The values obtained were corrected for self-absorption and expressed in terms of the weight of the precipitated benzidene sulphate. Statistically, the results are highly significant. We were interested in relative rather than absolute values and the values obtained for each of the applied substances should

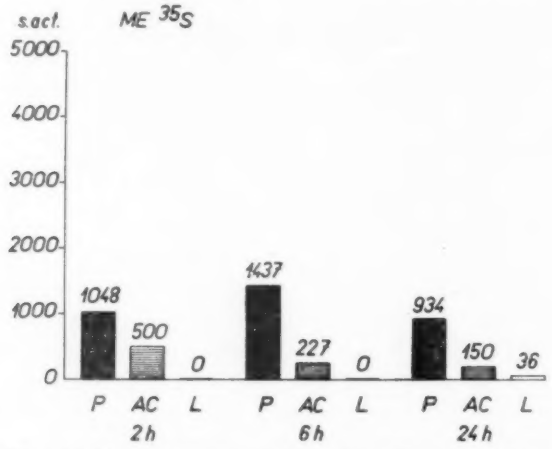

FIG. 1.—[35S]-DL-Methionine incorporation in proteinous ■, non-proteinous ▒, and lipidic □ fraction of rat brain 2,6 and 24 hours after i.p. injection. Abscissa—time, ordinate—impulses/min per mg precipitate of benzidine sulphate.

be considered separately. Our aim was to ascertain into what fraction of the brain, and at what rate, the ^{35}S from each of the substances was incorporated and for the sake of clearness, the results have been expressed graphically.

After evaporation on aluminium plates the samples of urine were measured with a G-M tube. Activities at the different times relative to the starting activity were thus obtained. After administration of methionine sulphone, the urine was also chromatographed and electrophoreted.

The time intervals in our investigation were chosen because maximum methionine incorporation in brain occurs after about 6 hr (GAITONDE and RICHTER, 1955). The extent of incorporation of the methionine derivatives was determined at the time corresponding to that at which there was full development of epileptic fits after the paroxysmal dose of methionine sulphoximine.

RESULTS

Control experiments showed that after intraperitoneal administration of [^{35}S]-methionine maximal incorporation into the protein fraction of brain occurred after about 6 hr (Fig. 1) suggesting lively metabolic activity of the brain. These results are similar to previous findings (GAITONDE and RICHTER, 1955).

The first oxidation product of methionine, methionine sulphoxide, gave rise to a slow incorporation of ^{35}S into proteins and lipoproteins of the brain (Fig. 2). Initially, i.e. after 2 and 6 hr, little had been incorporated; only after about 24 hr was a maximum reached and this subsequently declined. Elimination of this substance in the urine likewise attains a maximum at about 24 hr.

The highest oxidation product of methionine, the sulphone, was not

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incorporated into proteins or lipoproteins (Fig. 3). It was found in the so-called non-protein fraction where it reached its maximum at about 6 hr, after which the amount rapidly declined. The ^{35}S of methionine sulphone was eliminated in the urine mostly at the beginning, then the elimination gradually diminished. Chromatography and electrophoresis showed an enormously large spot, positive to ninhydrin,

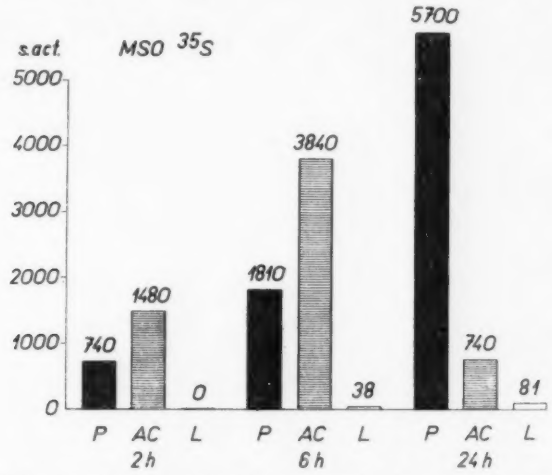

FIG. 2.—[^{35}S]-DL-Methionine sulphoxide incorporation in proteinous ■, non-proteinous ▒, and lipidic □ fraction of rat brain 2,6 and 24 hr after i.p. injection. Abscissa—time, ordinate—impulses/min per mg precipitate of benzidine sulphate.

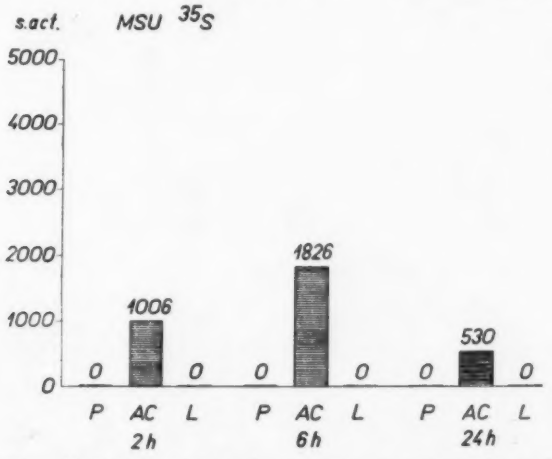

FIG. 3.—[^{35}S]-DL-Methionine sulphone incorporation in non-proteinous fraction ▒ of rat brain 2,6 and 24 hr after i.p. injection. Abscissa—time, ordinate—impulses/min per mg precipitate of benzidine sulphate.

containing ^{35}S in the sulphone position indicating that [^{35}S]methionine sulphone was eliminated mostly without change. It was not ascertained whether part of it was eliminated as the sulphate or if any was bound to glutamic acid (WINGO, SMITH and WOOD 1953).

The sulphur of [^{35}S]methionine sulphoximine was not incorporated into proteins or

lipoproteins (Fig. 4). It was contained in the non-proteinous fraction of the brain and its concentration reached a maximum at about 6 hr, i.e. at the time when full development of epileptic fits occurred. Elimination of the individual methionine derivatives in urine 2, 6 and 24 hr after i.p. injection is illustrated in Fig. 5.

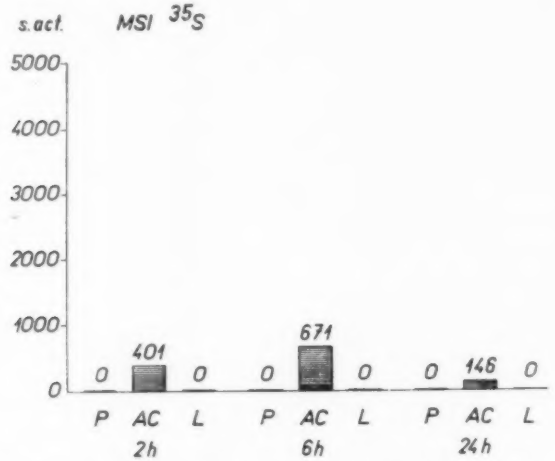

FIG. 4.—[35S]-DL-Methionine suplhoximine incorporation in non-protein fraction ▒ of rat brain 2, 6 and 24 hours after i.p. injection. Abscissa—time, ordinate—impulses/min per mg precipitate of benzidine sulphate.

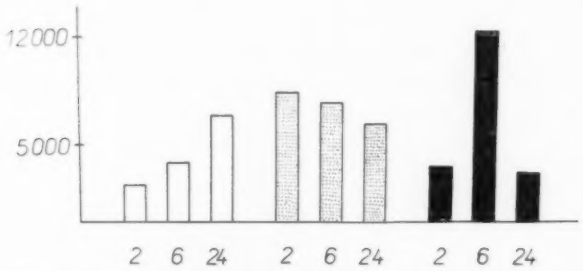

FIG. 5.—Elimination of 35S in urine of rats 2, 6 and 24 hours after i.p. application of [35S]-DL-methionine sulphoxide □, [35S]-DL-methionine sulphone ▒ and [35S]-DL-methionine sulphoximine ■. Abscissa—time, ordinate—impulses/min per mg of urine ash.

DISCUSSION

The incorporation of [35S]methionine into the brain was studied by GAITONDE and RICHTER (1955, 1956) and a picture of the movements of sulphur in brain was obtained. One hour after intracistal injection of [35S]methionine the 35S-labelled compounds in the acid-soluble fraction were methionine, 14 per cent, cystine and glutathione, 67 per cent and taurine, 19 per cent. In the proteins, 3 hours after intracistal injection, 76 per cent of the sulphur was bound to methionine and 24 per cent to cystine. The ratio between the two amino-acides eventually reached dynamic equilibrium. A very small amount of sulphur (5·7 per cent) in the form of cystine was bound to peptides by

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S–S links (GAITONDE and RICHTER, 1956). On intraperitoneal injection of [^{35}S]methionine into rats, the incorporation of this amino-acid into the brain proteins reached its maximum about 6 hours after administration. It is evident that the metabolic activity of the brain is high, a fact recently confirmed by the study of methionine incorporation into the cell microstructures of brain (CLOUET and RICHTER, 1959).

The first oxidation product of methionine, methionine sulphoxide which is held by DENT (1947) to be a natural oxidation product of methionine, presents another picture. The sulphur of this substance was incorporated into proteins and lipoproteins, but very slowly in contrast to methionine. This is probably because before incorporation occurs the molecule is reduced to methionine and actual incorporation becomes slower. We know from previous experiments that methionine sulphoxide relieves epileptic fits evoked by methionine sulphoximine much more slowly than does methionine. It may be that the efficacy of methionine sulphoxide *in vivo* is due to its reduction to methionine.

Methionine sulphone was found only in the acid-soluble fraction and was rapidly eliminated from the organism in the urine. It is probable that its reduction to methionine *in vivo* does not occur. The sulphur from it does not, therefore, become a component either of proteins or lipoproteins. Methionine sulphone evidently does not take part in metabolism and cannot substitute for methionine. This corresponds to the previous discovery that methionine sulphone cannot replace methionine in the diet of rats (BENNETT, 1941). The rapid elimination of methionine sulphone in urine agrees with the findings of WINGO, SMITH and WOOD (1953). It was not, however, our aim to determine in what form this substance leaves the organism.

From the fact that methionine sulphoximine (structurally similar to methionine and glutamine) was incorporated neither into proteins nor into lipoproteins, and that its highest level in the so-called non-protein fraction was reached after about 6 hours, i.e. at the time of full development of epileptic fits, one may conclude that the substance acts indirectly. This view is strengthened by the observation that methionine sulphoximine applied directly to the brain cortex of experimental dogs did not, during 8 hours, provoke any changes in the EEG (GERSHOFF and ELVEHJEM, 1951). The view that methionine sulphoximine could be incorporated into proteins in place of methionine, and so disturb brain metabolism (ROTH *et al.*, 1953), has not been supported by our experiments.

Previous work has shown that methionine sulphoximine inhibits protein synthesis *in vitro* (RYCHLÍK, ŠVEJCAR and ŠORM, 1955; RYCHLÍK and ŠORM, 1958) and that it influences the physiologically important reaction: acid + ammonia $\rightleftarrows$ glutamine, derangement of which leads to the onset of epileptic fits (KOLOUŠEK and JIRÁČEK, 1959).

It can therefore be concluded that methionine sulphoximine, without being incorporated into the proteins, affects enzyme systems concerned in brain metabolism.

SUMMARY

Methionine sulphoxide, methionine sulphone and methionine sulphoximine, labelled with ^{35}S, were prepared. The incorporation of ^{35}S from these substances into proteins and lipoproteins of brain, as well as its occurrence in the so-called non-protein fraction of the brain was compared with observations after the administration of [^{35}S]-methionine. Observations were carried out 2, 6 and 24 hours after intraperitoneal

injection of the above mentioned substances. The rate of elimination of these substances in urine was simultaneously followed.

[^{35}S]methionine sulphoxide was incorporated into proteins and lipoproteins of the brain much more slowly than methionine.

[^{35}S]methionine sulphone and [^{35}S]methionine sulphoximine were not incorporated into proteins or lipoproteins; they occurred only in the so-called non-protein or acid-soluble fraction of the brain.

A maximal content of [^{35}S]methionine sulphoximine in the non-protein fraction of the brain was found about 6 hours after i.p. administration of the paroxysmal dose of the substance, i.e. at the time when full development of epileptic fits took place.

Methionine sulphoximine, without being a component of proteins, affects brain metabolism.

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THE RELATION OF GAMMA-AMINOBUTYRIC ACID TO FACTOR I IN BRAIN EXTRACTS*

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CRYSTALS showing high Factor I activity (inhibition of spontaneous discharges by the crayfish stretch-receptor neurone) which were identified as γ-aminobutyric acid (GABA)‡ were obtained by BAZEMORE, ELLIOTT, and FLOREY (1957) when beef brain extracts were fractionated and the distribution of Factor I in the fractions was followed by the stretch-receptor assay. The fractionation procedure was not, however, fully quantitative so that this work did not show whether GABA could account for all the Factor I in brain. The total Factor I activity of whole brain did, nevertheless, correspond approximately to that to be expected from earlier and later GABA determinations by colorimetric procedures. Some comparative figures are given in a review by ELLIOTT and JASPER (1959).

More recently a number of other substances has been found to show strong Factor I activity (see ELLIOTT and JASPER, 1959). Estimations of GABA in rat cerebral cortex by a column chromatographic procedure indicate the presence of only about 200 μg per g of tissue (BERL and WAELSCH, 1958), whereas about 300 μg per g would be expected from Factor I assays (ELLIOTT and VAN GELDER, 1958). FLOREY and BIEDERMAN (1959) reported that the Factor I activity in inhibitory fibres from the crab is so high that it could not possibly be accounted for by GABA. MCLENNAN (1958, 1959) reported that sometimes no spot corresponding to GABA can be found on paper chromatograms from certain extracts from beef brain which show strong Factor I activity. Further, he obtained chromatographic evidence for the presence in his extracts of two substances which inhibited the response of the crayfish stretch-receptor neurone (MCLENNAN, 1959). One of these was definitely not GABA. The other substance behaved chromatographically like GABA but apparently gave a positive Sakaguchi reaction for the guanidino group. This led MCLENNAN to believe that this active substance was γ-guanidinobutyric and/or β-guanidinopropionic acid.

The present study was undertaken in an attempt to clarify the relationship of Factor I to GABA in brain by re-examining the observations of MCLENNAN. GABA was undoubtedly present in all brain extracts that we prepared and can account for all the Factor I activity in these extracts; guanidino compounds could not account for more than a trace of the total activity. The second active substance of MCLENNAN is most probably glutamate, which shows Factor I activity at low pH.

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‡ *The abbreviations used are:* GABA, γ-aminobutyric acid; GABOB, γ-amino-β-hydroxybutyric acid; GGBA, γ-guanidinobutyric acid; BGPA, β-guanidinopropionic acid.

METHODS

γ-Guanidinobutyric acid was kindly given us by Dr. F. IRREVERRE, and β-guanidinopropionic acid was obtained from the California Corporation for Biochemical Research.

Tissue extracts. 'FLOREY-MCLENNAN extract' was prepared from beef whole brain by the procedure of FLOREY and MCLENNAN (1955*b*). This procedure was developed empirically by them (1955*a*, 1955*b*) for the partial purification of Factor I before the activity of GABA was known. It involves heating the tissue, homogenizing it in water, dialysing, concentrating the dialysate and treating it with charcoal, with 5% perchloric acid, and with 10 volumes of ethanol and 5 volumes of ether; the supernatant fluid after these treatments is evaporated *in vacuo*, the residue is taken up with ethanol, adjusted to pH 5, dried, and extracted with water. The pH adjustment introduces approximately 100 m-equiv/l. of Na ions; as will be discussed later, this influences the chromatographic behaviour of the extracts.

'Water extract' was the clear, unconcentrated supernatant fluid obtained by homogenizing whole brain in 4 vol. of water, heating in boiling water, acidifying with acetic acid to about pH 5 (for better precipitation of protein), and centrifuging.

Chromatography. Whatman No. 4 paper, 46 × 57 cm, was used throughout. Descending chromatograms were run, with the solvents mentioned later, at room temperature in 'Chromatocab' cabinets (Research Specialities Co). When extracts were desalted, this was done with the apparatus of STEVENS, SMITH and JEPSON (see SMITH, 1958). When a large volume (1 ml) had to be applied as a spot, the sample was evaporated dry *in vacuo*. The residue was redissolved in 0·1 ml and two rinsings of 0·05 ml of water.

Staining. Spots were located by dipping the chromatograms in 0·25% ninhydrin in acetone, drying, and heating briefly in an oven at 100°. The Sakaguchi reagents were applied according to ACHER and CROCKER (1952). In some cases the relative positions of the zones occupied by GABA and the guanidino compounds could be directly observed by applying the Sakaguchi reagents to papers which had been treated with ninhydrin. In some cases this procedure was not satisfactory and separate strips had to be stained with the different reagents.

Determination of GABA. Chromatograms of a synthetic amino acid mixture and of the two types of brain extract with *n*-butanol–ethanol–acetic acid–water* (8:2:1:3 v/v) run for 12 hr gave good separation of the spot corresponding to GABA from other ninhydrin-stainable spots. On running such chromatograms in the second dimension with phenol–water (80:20 w/w) there was no sign of resolution of the spot which contained GABA into more than one spot (Fig. 1). The following method involving one-dimensional chromatography was therefore used for the determination of GABA. Amounts of extract which contained 1–20 μg of GABA were applied to paper as spots and chromatographed with butanol–ethanol–acetic acid–water for about 12 hr. The paper was dipped in 0·25% ninhydrin in acetone and heated for 3 min in an oven at 100°. The zone that corresponded to GABA was identified by running, on the same sheet, a spot containing extract plus a known amount of added GABA. The stained zone thus identified was cut out and treated with 1 ml of freshly prepared 0·5% ninhydrin in 80% (v/v) ethanol at room temperature for 15 min then 3 ml of 70% (v/v) acetone were added and, after 15 min, the fluid was transferred to a graduated tube. The paper was re-extracted for 15 min with a further 3 ml of 70% acetone and the combined fluids were made up to 8 ml with 70% acetone. The absorption of this solution was measured at 575 mμ in a Beckmann spectrophotometer against similarly prepared ninhydrin–ethanol–acetone extract from a part of the paper which showed no colour. Standard solutions of GABA, alone and in the same spot as tissue extract, were applied to the same sheet of paper, the relevant spots were extracted and developed, and the absorption was measured. The recovery of 6–10 μg GABA applied with 25–100 μl of extract was 95–106 per cent.

Assay of Factor I activity. The assay of Factor I activity, *i.e.* inhibition of the crayfish stretch receptor neurone, was carried out as described by ELLIOTT and FLOREY (1956). In the procedure routinely used in this laboratory (ELLIOTT and VAN GELDER, 1958) the saline solution used for diluting extracts for the assay contains 5 m-moles glutamate and is buffered to pH 6·5 with tris-maleate. This solution which contains, (m-moles) NaCl, 186; KCl, 49; $MgCl_2$, 2·4; $CaCl_2$, 12·1; glutamate 5; and tris-maleate 10; was used in obtaining results shown in Table 1 but, as will be discussed below, the composition of the dilution solution was varied with respect to glutamate, pH, and buffer for specific

* This solvent was used for the determinations, in preference to the ethanol-free mixture which was used in other experiments, because it seemed to minimize salt effects. There was, however, little or no difference between separations obtained with these two solvents.

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purposes. The Florey-McLennan and water extracts, neutralized, were assayed without special adjustment of salt content since the assay procedure involved the dilution of these extracts at least ten fold with the appropriate saline solution. For the detection of Factor I on chromatograms relevant portions of the papers were eluted with crayfish saline and the eluate was assayed. In a number of cases the extract was reassayed on different stretch receptor preparations. The widest difference from the mean of such replicate assays was 11%.

Factor I is present in tissue in an 'occult' and a 'free' form (ELLIOTT and FLOREY, 1956). The factor in the extracts studied in the present work was all free since the tissue was extracted with hypotonic solution (water) and, in most cases, heated.

TABLE 1.—COMPARISON OF THE γ-AMINOBUTYRIC ACID CONTENT AND THE FACTOR I ACTIVITY OF BRAIN EXTRACTS

Animal	Extraction	GABA* (μg/ml extract)	Factor I† (as μg GABA/ml extract)
Beef	Florey-McLennan	192 (4) 180–202	190 (3) 178–206
Beef	Water	50 (4) 46–58	55 (3) 49–62
Cat	Florey-McLennan	206 (3) 178–232	200†
Cat	Water	70 (4) 64–75	77 (2) 75–78
Rat	Water	72 (4) 68–74	71 (2) 70–71
Rat	Water unheated‡	76 (3) 72–80	69†

* The figures for GABA indicate the mean (number of determinations in parentheses) and range of results.

† Each recorded figure for Factor I is the mean of a series of determinations on a stretch receptor preparation. Where several results are given for one extract these were obtained with different receptor preparations.

‡ This extraction was made with ice-cold water and the extract was not heated.

RESULTS

GABA content and Factor I activity. Extracts were prepared by the FLOREY-MCLENNAN method, twice from beef brain and four times from cat brain. Simple water extracts were prepared, twice from beef brain, twice from cat brain and twelve times from rat brain. Samples of each of these extracts were subjected to two-dimensional chromatography with *n*-butanol–acetic acid–water (12:3:5 v/v)* and phenol–water (80:20 w/w), solvents used by MCLENNAN, followed by ninhydrin staining, and in every case spots corresponding to GABA were obtained. Examination of the two-dimensional chromatograms indicated that one-dimensional chromatography with the butanol–acetic acid–water was sufficient to separate and identify GABA. Such one-dimensional chromatograms were performed on the two kinds of brain extracts many times and always indicated the presence of GABA.

The concentrations of GABA in a number of brain extracts were determined by the procedure described under *Methods* and compared with Factor I assays. The results (see Table 1) show that GABA could account for the Factor I activity in all cases.

All the extracts listed in Table 1 were tested as soon as the preparation was made.

* This mixture has approximately the same composition as the organic layer of butanol–acetic acid–water 4:1:5 (SMITH, 1958) which was actually used by MCLENNAN.

MCLENNAN (1959) suggested that the natural form of Factor I might be a GABA derivative which is very readily convertible to GABA and shows about the same activity as GABA toward the crayfish stretch receptor neurone. To avoid such a conversion, a water extract of rat brain was prepared in the cold (never heated) and kept cold until a sample was applied to the paper for GABA determination. Again the Factor I activity found by assay and the GABA content determined chromatographically corresponded rather closely (Table 1).

Factors affecting the chromatographic behaviour of GABA. The occasional failure by MCLENNAN to detect GABA chromatographically in Factor I-active extracts is difficult to explain. It is well known that the chromatographic behaviour of a substance may be affected by accompanying material. We have observed, for instance, that the R_F of pure GABA with butanol–ethanol–acetic acid–water was about 0·4 but GABA mixed with 1 ml of Florey–McLennan extract had an R_F of about 0·55. (This effect did not occur if the Florey–McLennan extract was desalted before the chromatography). If a relatively large volume (0·5–1·5 ml) of Florey–McLennan extract is applied for bidimensional chromatography, the GABA spot does not separate from a neighbouring spot; with a smaller amount of extract, separation is clear. It is possible that MCLENNAN's occasional negative findings depended on some such effect. We cannot, however, definitely exclude the possibility of the occasional presence in brain extracts of a substance which is derived from and can be converted to GABA without change in its Factor I activity.

With water-saturated butanol as chromatographic solvent, MCLENNAN (1959) found that the R_F of all Factor I activity was about 0·25, whereas that of GABA was less than 0·05. This would seem to indicate that the activity could not be due to GABA. We find that the R_F value of pure GABA with water-saturated butanol is about 0·08 to 0·12 but, when GABA is added to a Florey–McLennan extract, two ninhydrin-staining zones become accentuated. These two zones are found also with extract without added GABA. The R_F of one of these is about the same as for pure GABA; the R_F of the second zone increases from about 0·2 to 0·3 (sometimes even to 0·4) as the amount of extract applied as a spot is increased from 10 μl to 200 μl. These observations are illustrated in Fig. 2. Factor I activity was found in both these ninhydrin-staining zones and not elsewhere. With desalted extract only the first zone is obtained. The first and second zones have the same R_F value of 0·8 when rechromatographed in the second dimension with phenol–water. It seems that GABA exists in more than one form (perhaps open or cyclic or ionized in different ways) in neutral butanol–water. It is evident that the chromatographic behaviour of these forms can cause confusion.

Guanidino compounds. When MCLENNAN(1959) subjected his extracts to paper chromatography with various butanol–acid–water solvents or phenol–water he found that material eluted from two separate zones, A and B, showed Factor I activity and he concluded that neither of these was due to GABA. Zone B corresponded to the place where GABA is found but, because this zone was stainable with the Sakaguchi reagent, MCLENNAN tentatively concluded that the Factor I activity of this zone was due to a guanidino compound, γ-guanidinobutyric acid (GGBA) or β-guanidinopropionic acid (BGPA), substances which are known to exert this activity (EDWARDS and KUFFLER, 1959). The active substance in zone A was not identified.

We have confirmed the finding that, if sufficient Florey–McLennan extract of beef brain is applied in a two-dimensional chromatogram (butanol–acetic acid–water,

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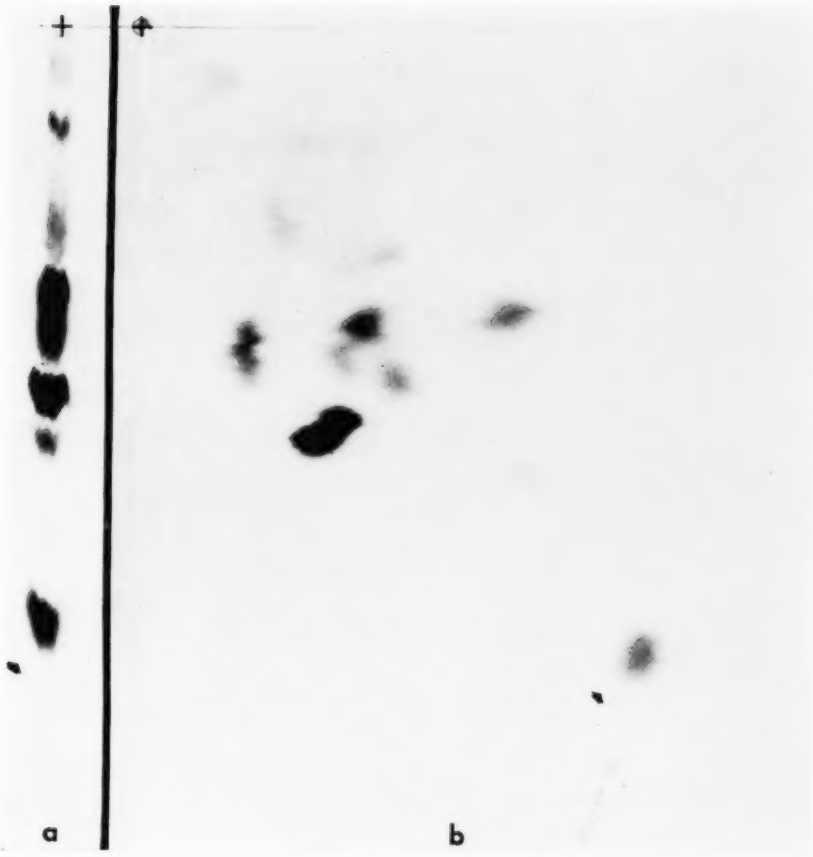

FIG. 1—Typical chromatograms of ninhydrin-positive material in brain extract. (a) One-dimensional chromatogram of 50 μl of water extract of beef brain with butanol–ethanol–acetic acid–water (12 hr); (b) two-dimensional chromatogram of 50 μl of the water extract with butanol–ethanol–acetic acid–water in the vertical direction (12 hr) and phenol–water in the horizontal direction (8·5 hr). When GABA (10 μg) was applied at the same point as extract, the spot indicated by the arrow in each chromatogram, and only this spot, was accentuated. Similar results were routinely obtained with extracts from brains of other animals and with Florey–McLennan extracts.

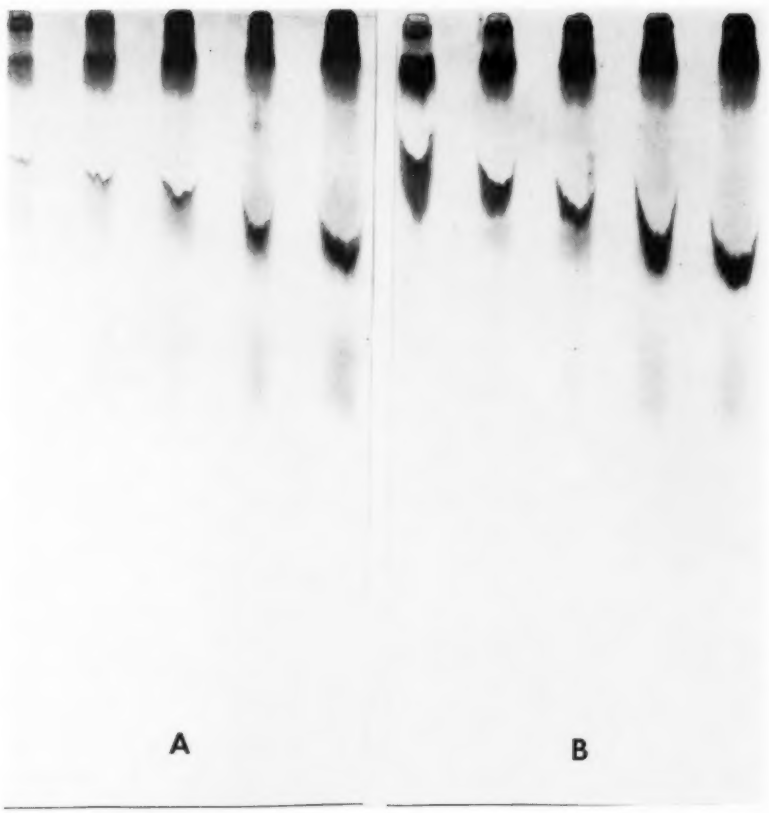

FIG. 2.—Chromatographic behaviour of GABA in Florey–McLennan beef brain extract with water-saturated *n*-butanol as solvent (running time, 9 hr). (A) From left to right: 10, 25, 50, 100, 200 μl of extract alone; (B) the same as (A) but with 50 μg of GABA added in each case. The bottom lines represent the solvent front.

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phenol–water) two zones are obtained which will stain faintly with Sakaguchi reagents. One of these is presumably due to GGBA, the other to arginine. When GABA, GGBA, or BGPA was added to the extract to be chromatographed, the ninhydrin and Sakaguchi stains showed that all three substances ran to about the same spot. Complete separation of the three substances could be obtained in a one-dimensional chromatogram with water-saturated butanol as solvent run for 48 hr (Fig. 3). Florey-McLennan

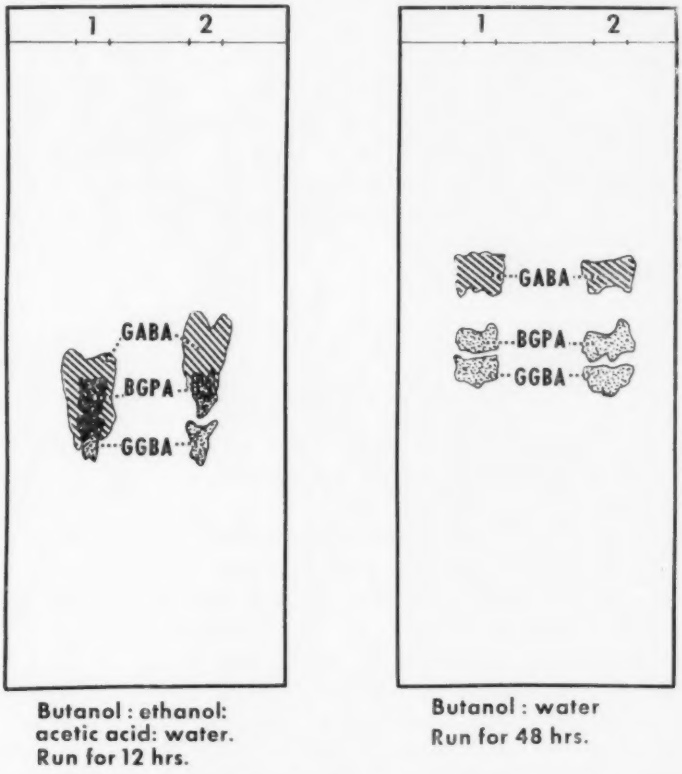

FIG. 3.—Chromatographic behaviour of GABA and guanidino compounds in beef brain extracts. The drawings indicate the positions of actual spots developed with ninhydrin and Sakaguchi stains on the same paper (spots due to other substances not shown). (1) Florey–McLennan extract; (2) water extract—one ml of each with 10 μg of GGBA and 10 μg BGPA added.

extract and simple water extract were applied to paper as short bands and chromatographed with butanol–water for 48 hr; strips were cut off and stained with ninhydrin and Sakaguchi reagents and the zones on the remainder of the paper that contained GABA and guanidino compounds were eluted and biologically tested.* Factor I activity was found only in the GABA zone. Though GGBA and BGPA are known to be active, the intensities of our Sakaguchi-stained bands indicated the presence of only about 2–4 μg of GGBA, and no BGPA, per ml of extract.† As much as 30–100 μg

* After 48 hr only the spot due to GABA with the lower R_F remained on the paper; the other GABA spot which appears with the butanol–water solvent had been carried off the end of the paper.

† Chromatograms of undesalted Florey–McLennan extract sometimes give a faint Sakaguchi-staining spot which could be mistaken for BGPA; after desalting this spot does not appear.

of GGBA would have been required to demonstrate Factor I activity due to GGBA in the eluate from the paper, but Factor I activity could be detected in the eluate from the spot obtained from only 0·1 ml of the extract which would contain only 0·2–0·4 μg of the guanidino compound. We conclude that the activity in MCLENNAN's zone B is due almost entirely to GABA but that this zone may stain somewhat with Sakaguchi reagent because GGBA runs to the same zone under MCLENNAN's conditions. GGBA is present in brain but in much smaller concentration than is GABA (IRREVERRE *et al.*, 1957) and, since it is less active than GABA, its contribution to the total Factor I activity of the extract would be very small.

Glutamate and Factor I activity. ELLIOTT and VAN GELDER (1958) noted that glutamate can exert variable effects on the crayfish stretch receptor and they therefore added an excess of glutamate to all fluids used in the assay to overcome variations in the amounts of glutamate that may be present in brain extracts. When eluates from paper chromatograms were made with glutamate-containing saline, and the solutions were buffered as is customary in this laboratory, no second zone with Factor I activity corresponding to MCLENNAN's zone A could be found. When, however, unbuffered solutions without added glutamate were used, eluates from zone A inhibited the stretch receptor response and MCLENNAN's observation was thus confirmed.

Zone A corresponded to the position to which glutamate ran with all the five solvents used by MCLENNAN (1959). At neutral pH, glutamate is sometimes excitatory, sometimes inhibitory; often the first application will cause excitation or inhibition and later applications have no effect. But at relatively low pH, about 5·5, glutamate solutions nearly always block the stretch receptor, the concentration required being between 5 μg and 30 μg per ml.

It could be concluded, therefore, that the active substance of MCLENNAN's fraction A, obtained from zone A, is glutamate which behaves as Factor I under some conditions, particularly at low pH. GABA was inhibitory at all pH values studied. With stretch receptor preparations adapted to glutamate, glutamate does not obviously affect the action of GABA, qualitatively or quantitatively.

γ-Amino-β-hydroxybutyric acid. HAYASHI (1959) and his co-workers indicated that γ-amino-β-hydroxybutyric acid (GABOB) is a more potent inhibitory agent than GABA in the central nervous system. OHARA et al. (1959) reported evidence, obtained by high voltage electrophoresis and chromatography, for the presence of free GABOB in mammalian brain—nearly 500 μg per g in beef temporal lobe. We have not been able to obtain evidence for the presence of GABOB in a water or Florey–McLennan extract of beef brain, or in an alcoholic extract prepared from cat brain according to OHARA, by two-dimensional paper chromatography (butanol–acetic acid–water, phenol–water) and staining with ninhydrin or with the copper nitrate–ninhydrin method of LARSEN and KJAER (1960). With authentic GABOB, both of these staining methods showed the two spots mentioned by OHARA, one of which is due to a cyclic derivative of GABOB; by the LARSEN–KJAER method the straight chain form of GABOB was stained brown and the cyclic form yellow. Correspondingly stained areas were not detectable on chromatograms of the extracts. Amounts corresponding to 5 μg per g brain could have been clearly detected by our method.

DISCUSSION

There is some confusion about the use of the term 'Factor I'. MCLENNAN uses the

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term to describe an extract which can exert a number of biological effects and seems to assume that all the effects are due to one factor and to conclude that a substance which does not exert all these effects is not 'Factor I'. We consider it probable that a brain extract would contain a variety of substances which could exert a variety of effects. We use the term Factor I only for natural substances which inhibit the responses of the crustacean stretch receptor neurone. Such substances may exert a number of other effects. GABA is a Factor I because it occurs in brain and inhibits the stretch receptor neurone. GABA also exerts marked effects on the mammalian nervous system and on a variety of test objects (see ELLIOTT and JASPER, 1959). The presence of GABA can account for many but apparently not all (MCLENNAN, 1957, 1959; HONOUR and MCLENNAN, 1960) of the effects of Florey–McLennan extracts on preparations other than the crustacean stretch receptor. It is our thesis that GABA probably accounts for all, or almost all, of the Factor I activity of brain extracts assayed at the usual pH 6·5.

Our experiments convince us that GABA is present, and responsible for the Factor I activity, in all brain extracts. (Preliminary work by B. BOLLARD shows agreement between figures for GABA, in brain extracts, obtained by chromatographic methods with two different stains and by an enzymic method). There does not seem to be any way that an extract prepared by the Florey–McLennan method could fail to contain GABA since it is well known that there are large amounts of this substance free in brain and the method includes no step that is likely to remove much GABA; nor is there any reason to suppose that the Florey–McLennan extraction is less likely to degrade a hypothetical labile derivative of GABA than is simple water or ethanol extraction.

γ-Aminobutyryl choline and γ-guanidinobutyric acid as well as GABA are present and free in brain and exert inhibitory actions. But GABA is the most potent of these in inhibiting the crayfish stretch-receptor neurone and the amounts of GABA present in whole brain extracts can, within the limits of accuracy of the methods, account for all this activity in these extracts. We consider it likely, however, that neuro-inhibitory substances other than GABA may exist in brain but that these may not show Factor I activity, i.e., they may not affect the crustacean stretch receptor neurone.

A comparative study, now in progress, of the Factor I activity and the GABA content, chemically and enzymically determined, in various regions of the brain should disclose any special locations where GABA cannot account for Factor I. FLOREY and BIEDERMAN (1959) find that inhibitory fibres of the crab's claw apparently contain a substance which is far more active as Factor I than is GABA. Such a substance may be present, though not yet detected, in special regions of the mammalian nervous system.

The inhibitory activity of glutamate at low pH is of probable physiological interest. For instance, the depression of the nervous system that is associated with acidosis might be connected with development of inhibitory activity by the glutamate present in the brain.

Addendum. After this work was completed, a more recent paper by MCLENNAN (1960) came to our attention. In this study McLennan obtained, by ion exchange procedures, preparations of his fractions A and B with increased specific activity but still undetermined chemical constitution. He was unable to produce a ninhydrin reaction with these fractions. However, tables in this and the previous (MCLENNAN, 1959) paper contain figures for the R_F values of the fractions A and B which are approximately those that we find for glutamic acid and GABA. The specific

activity which is given for the purified Fraction A is higher than we have observed with glutamic acid. The activity of the latter substance is, however, highly variable.

SUMMARY

(1) Chromatographic evidence for the presence of γ-aminobutyric acid (GABA) was always obtained in extracts prepared by two different methods from the brains of cattle, cats, and rats. The amounts of GABA present, chromatographically determined, could account for the Factor I activity in every case.

(2) The presence of salts in extracts greatly affects the chromatographic behaviour of GABA; with neutral water-saturated butanol as solvent, GABA in salt-containing Florey-McLennan extracts runs to two separate zones.

(3) γ-Guanidinobutyric and β-guanidinopropionic acids behave chromatographically like GABA with some solvents but can be separated from it. No β-guanidinopropionic acid could be detected in brain extracts and the slight amount of γ-guanidinobutyric acid present could not account for more than a trace of the Factor I activity of whole brain.

(4) Glutamate exerts variable effects on the crayfish stretch receptor neurone. At low pH, with receptors not adapted to glutamate, this amino acid inhibits activity, that is, it behaves as Factor I.

(5) Eluates from two zones of butanol–acid–water chromatograms of brain extract show Factor I activity if the dilution media are unbuffered and contain no glutamate. One of these zones is due to GABA. The other is almost certainly due to glutamate.

(6) No γ-amino-β-hydroxybutyric acid was detected in brain extracts.

(7) It is recommended that the term 'Factor I' be used only for natural substances that inhibit the crustacean stretch receptor neurone and not for extracts which may contain a variety of pharmacologically active substances.

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AN ENZYME FORMING VASODILATOR POLYPEPTIDES IN HUMAN CEREBROSPINAL FLUID

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THE formation of vasodilator polypeptides has been suggested as the mechanism by which functional vasodilatation is brought about in glandular organs (HILTON and LEWIS, 1956, 1957, 1958). Such polypeptides have been given the generic name of plasma kinin, the best known being bradykinin. Bradykinin was discovered by ROCHA E SILVA, BERALDO and ROSENFELD (1949). It is a nona-peptide and was recently isolated (ELLIOTT, LEWIS and HORTON, 1960) and synthesized (BOISSONNAS, GUTTMANN, JAQUENOUD, KONZETT and STÜRMER, 1960).

CHAPMAN and WOLFF (1958, 1959) reported the presence of an enzyme which forms plasma kinin in the cerebrospinal fluid of subjects with various neurological disorders and with schizophrenia. They concluded that the plasma kinin-forming system is activated during the altered metabolism which occurs with active diseases of the central nervous system. In contrast, HAMBERG (1960) found only traces of enzymic activity in the cerebrospinal fluid of subjects suffering from neurological diseases. The controversial nature of the issue made it necessary to re-examine this problem.

The results of the present experiments are in agreement with those of HAMBERG. The enzyme content of the cerebrospinal fluid from schizophrenic patients, did not differ from that of patients without progressive disease of the central nervous system nor from that of patients with active neurological disorders. In all three groups slight plasma kinin formation could be detected in the cerebrospinal fluid of some of the patients.

METHODS

The cerebrospinal fluid was obtained by lumbar puncture, except that in 4 patients undergoing a ventriculogram ventricular cerebrospinal fluid was taken (Table 3). The samples were collected in siliconed tubes and immediately frozen and stored at $-10°$. They were usually tested within 48 hr and always within 5 days of collection. Six specimens (Nos. 21, 26, 27, 29 and 30 in Table 1 and No. 24 in Table 2) were tested 1–2 hr after collection. The samples were tested on the isolated uterus preparation of rats (200–250 g) into which 0·2 mg stilboestrol had been injected subcutaneously, 12–18 hr previously. This treatment assured an oestrous uterus which is more sensitive to bradykinin than the dioestrous or anoestrous uterus which was used by CHAPMAN and WOLFF (1959). The uterus was suspended in 15 ml of oxygenated de Jalon solution at 29–30°, the sample was added to the bath and the response compared with the response to a standard preparation of bradykinin, or to 5-hydroxytryptamine. Forty-two samples of cerebrospinal fluid were tested in this way for the presence of a plasma kinin or 5-hydroxytryptamine. All samples were tested for plasma kinin-forming enzyme activity. Immediately after thawing they were incubated with the pseudoglobulin fraction of plasma proteins for 2 min at 30° and the uterine stimulating activity of the incubated mixture was estimated in terms of a standard bradykinin. *Pseudoglobulin* was prepared from either dog or ox plasma and

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consisted of the fraction of plasma protein precipitated by $(NH_4)_2SO_4$ (33–46% saturation). This precipitate was dialysed for 48 hr against running tap water and the final solution (one third of the original volume) was freeze-dried in 2 ml ampoules and stored at −10°. The most suitable concentration for the estimation of enzyme activity was 0·1 ml of pseudoglobulin solution (containing 10% protein) per ml of sample. *Standard bradykinin* was prepared by the procedure of ELLIOTT, LEWIS and HORTON (1960) from trypsin and ox globulin. It contained 2·5 μg/mg pure peptide.

TABLE 1.—PATIENTS WITH SCHIZOPHRENIA

Sample No.	Age (yr)	Sex	Length of history (yr)	Treatment	Enzyme activity (μg standard bradykinin/ml)	Inhibition*
1	47	M	1	Trifluoperazine	0	0
2	40	F	10	Trifluoperazine	0	0
3	32	F	9	Chlorpromazine	0	0
4	62	M	23	Ephedrine	1·0 repeat 0	0
5	59	F	24	Chlorpromazine	0·5	0
6	29	F	8	Chlorpromazine, Trifluoperazine	0	0
7	30	F	6	Chlorpromazine, Trifluoperazine	0	0
8	60	F	21	Tetrabenazine	0	0
9	51	F	10	Chlorpromazine	0	0
10	48	M	15	Chlorpromazine	0	0
11	25	M	3	Chlorpromazine	0	0
12	38	M	16	Prochlorperazine	0	marked
13	53	F	8	Chlorpromazine	0·3	0
14	42	F	17	Chlorpromazine	0	0
15	50	F	12	Chlorpromazine	0·3	0
16	68	F	30	None	0	moderate
17	33	M	1½	None	0	0
18	70	F	37	None	0·4	0
19	62	M	39	None	5·0 repeat 0	0
20	34	M	1	None	0	slight
21	54	F	4	None	0	moderate
22	43	M	24	None	0	0
23	50	M	20	None	0	0
24	52	M	35	None	0	0
25	66	F	32	None	0	0
26	52	F	14	None	0	moderate
27	54	F	3	None	0	0
28	54	F	26	None	0	v. marked
29	71	F	33	None	0	0
30	75	F	17	None	0	0
31	54	F	6	None	0	0

* Inhibition represents inhibition of isolated rat uterus to subsequent dose of bradykinin.

Patients

Cerebrospinal fluid was collected from three groups of patients. Group 1: Schizophrenia—31 patients in whom the diagnosis of schizophrenia was unequivocal were selected for testing. Details of the case histories are given in Table 1. Group 2: Patients without progressive disease of the central nervous system. This group contained 24 patients, 5 of whom were undergoing spinal anaesthesia for abdominal operation; none of them had suffered pain in the days preceding the operation. The clinical conditions of the other patients are shown in Table 2. None of these patients had an abnormal

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CSF and no other neurological abnormality could be found beyond those indicated in the diagnosis. Group 3: Patients with active neurological disease. This group consisted of 23 patients, whose neurological condition is shown in Table 3.

RESULTS

Forty-two of the 78 samples were tested on the rat uterus for bradykinin or 5-hydroxytryptamine-like activity. Only two samples, Nos. 10 and 24 (Table 2) caused contraction of the uterus. The nature of this stimulant was not determined although

TABLE 2.—PATIENTS WITHOUT PROGRESSIVE DISEASE OF THE CENTRAL NERVOUS SYSTEM

Sample No.	Age (yr)	Sex	Condition	Treatment	Enzyme activity (μg standard bradykinin/ml)	Inhibition*
1	42	F	Spinal anaesthesia	None	0	0
2	60	M	Spinal anaesthesia	None	0	0
3	49	F	Spinal anaesthesia	None	0	0
4	66	F	Spinal anaesthesia	None	0	0
5	60	F	Spinal anaesthesia	None	0	0
6	27	M	Psychopath	None	0·5	0
7	35	M	Recovered depression	None	0·4	0
8	19	F	Psychopath	None	0·5	moderate
9	32	M	Vertigo	None	0	0
10	45	M	Back pain	None	0	0
11	36	F	Back pain	None	0	0
12	22	F	Back pain	None	0	0
13	42	M	Epilepsy	Phenobarbitone, Primidone	0·6	0
14	21	F	Epilepsy	Primidone	1·0	moderate
15	25	F	Epilepsy	Primidone, Tridione	0·5	0
16	8	F	Epilepsy	Phenobarbitone	0	0
17	13	F	Epilepsy	Primidone	0·3	0
18	27	M	Epilepsy	Primidone	0	0
19	28	M	Epilepsy	Primidone	0	0
20	29	F	Epilepsy	Primidone	0	0
21	57	F	Epilepsy	Phenobarbitone	0	0
22	33	M	Epilepsy	Phenobarbitone	0	0
23	30	M	Epilepsy	Phenobarbitone	0	0
24	25	F	Epilepsy	None	0	0

* Inhibition represents inhibition of isolated rat uterus to subsequent dose of bradykinin.

the contraction produced by sample No. 24 was abolished by 2-bromolysergic acid diethylamide (BOL) suggesting that it was due to 5-hydroxytryptamine.

The addition of some samples of cerebrospinal fluid to the organ bath, caused a reduction in the response of the uterus to subsequent additions of standard bradykinin. The effect was obtained with 10 samples, as shown in the Tables, and it varied in intensity.

In the majority of samples of cerebrospinal fluid from the three groups of patients no plasma kinin-forming enzyme activity could be detected, but all groups contained some samples which were active. Enzyme activity was present in 6 out of the 31 schizophrenics, in 8 out of the 24 patients without progressive disease of the nervous system and in 7 out of the 23 patients with such disease. Only one of the four samples

of the ventricular cerebrospinal fluid showed slight enzyme activity. Fig. 1 shows the activity in samples No. 15 (Table 2) and No. 2 (Table 3), and the absence of activity in 5 other samples. Samples 4 and 19 (Table 1) showed the greatest enzyme activity of the schizophrenic group. When samples were taken from these two patients several months later they showed no enzyme activity in spite of the fact that the clinical condition of the patients was apparently unchanged.

TABLE 3.—PATIENTS WITH ACTIVE DISEASE OF THE CENTRAL NERVOUS SYSTEM

Sample No.	Age (yr)	Sex	Diagnosis	CSF Protein (mg %)	Enzyme activity (μg standard bradykinin/ml)	Inhibition*
1	54	M	Cerebral tumour	35	3·0	0
2	47	F	Cerebral tumour	40	1·5	slight
3	63	M	Cerebral tumour	30	0·3	0
4	28	M	Cerebral tumour	55	0	0
5	53	M	Cerebral tumour	50	0	0
6	38	M	Cerebral tumour	40	0	0
7	67	F	Cerebral tumour	60	0·5	0
8	35	F	Cerebral tumour	70	0	0
9	61	M	Cerebral tumour	—	0†	0
10	12	M	Cerebeller tumour	—	0·3†	0
11	25	M	Cerebeller tumour	—	0†	0
12	44	M	General paresis dementia	60	0·4	0
13	38	F	Chromaphobe adenoma of pituitary	40	0·6	0
14	58	M	Dementia	20	0	moderate
15	7 months	F	Hydrocephalus	—	0†	0
16	69	F	Multiple sclerosis	65	0	0
17	52	F	Polyneuritis	85	0	0
18	64	M	Parkinsonism	40	0	0
19	21	F	Post-traumatic paresis and headaches	35	1·0	0
20	74	M	Brainstem lesion	80	0	0
21	25	F	Lumbar disc lesion	35	0	0
22	21	F	Lumbar disc lesion	40	0	0
23	47	F	Lumbar disc lesion	—	0	0

* Inhibition represents inhibition of isolated rat uterus to subsequent dose of bradykinin.
† Ventricular fluid.

DISCUSSION

The present experiments fail to substantiate the claim of CHAPMAN and WOLFF (1958, 1959) that there is a correlation between the presence of a plasma kinin-forming enzyme in the cerebrospinal fluid and certain neurological disorders. The proportion of patients showing enzymic activity in the cerebrospinal fluid was the same whether or not they were suffering from progressive nervous disease. It is not possible to explain this discrepancy.

In determining enzymic activity in cerebrospinal fluid care must be taken to avoid the presence of traces of blood in the sample of cerebrospinal fluid and to avoid using too much of the substrate, pseudoglobulin. Blood contains at least two plasma kinin-forming enzymes (FREY, KRAUT and WERLE, 1950). Traces of blood in the cerebrospinal fluid, which might occur through bleeding during lumbar puncture, would be

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sufficient to give a spurious appearance of enzymic activity in the cerebrospinal fluid. Such an appearance could also result from the use of too much globulin as substrate, since it too contains small amounts of the plasma kinin-forming enzyme (LEWIS, 1960). We do not know whether these sources of error were fully excluded in the experiments of CHAPMAN and WOLFF.

These authors suggest that the dilatation of the cerebral vessels which is known to accompany central nervous activity results from the action of a vasodilator polypeptide produced by the plasma kinin-forming enzyme and that with increased nervous

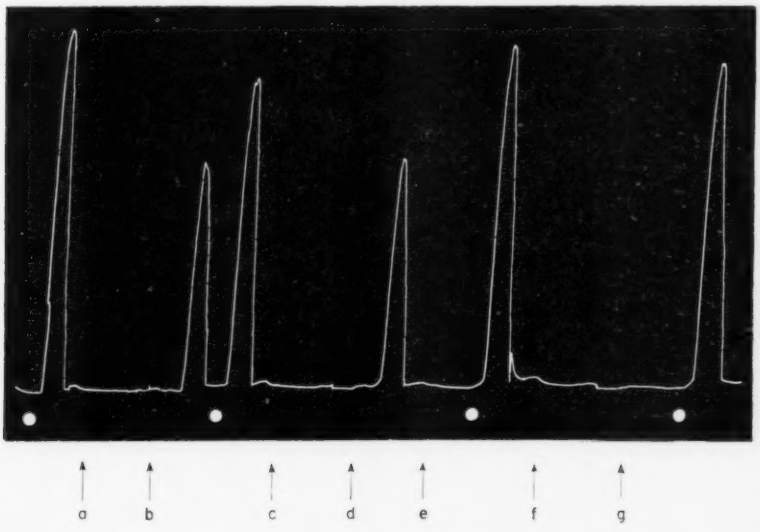

FIG. 1.—Responses of the isolated rat uterus to 0·5 μg bradykinin at (0); at the arrows, addition of an incubated mixture of 0·1 ml pseudoglobulin solution with 1 ml of (a) sample No. 14 (Table 3); (b) No. 15 (Table 2); (c) No. 16 (Table 2); (d) No. 2 (Table 3); (e) No. 6 (Table 2); (f) No. 17 (Table 1); and (g) No. 6 (Table 1).

activity, which they assume occurs during the nervous disorders, this enzyme escapes into the cerebrospinal fluid. Even if the vasodilatation in the central nervous system during increased activity is brought about in this way it is unlikely that the enzyme would find its way from the brain tissue into the cerebrospinal fluid.

On the other hand, the plasma kinin-forming enzyme found in the cerebrospinal fluid may originate from the choroid plexus. The theory that functional vasodilatation in glandular tissue is brought about by the escape of a plasma kinin-forming enzyme (HILTON and LEWIS, 1957) may also apply to the choroid plexus which is generally regarded as a tissue actively secreting cerebrospinal fluid. By analogy with the secretions of other glandular tissues, it would therefore not be surprising if cerebrospinal fluid were to contain plasma kinin-forming enzyme as well. However, if the enzyme were a product of secretion from the choroid plexus one would expect its concentration to be higher in the cerebrospinal fluid of the cerebral ventricles than in that of the lumbar subarachnoidal space. This was found not to be so.

SUMMARY

Samples of human cerebrospinal fluid were assayed for the presence of an enzyme which forms vasodilator polypeptides (plasma kinins) when incubated with plasma globulins.

A small amount of enzymic activity was detected in 21 out of 78 samples of cerebrospinal fluid.

The samples of cerebrospinal fluid containing enzymic activity were found to be randomly distributed amongst patients without nervous disorders, patients with progressive disease of the nervous system and schizophrenics. The claim of CHAPMAN and WOLFF (1958, 1959) that there is a relationship between the presence of a plasma kinin-forming enzyme in cerebrospinal fluid and certain neurological disorders and schizophrenia could not be substantiated.

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METABOLIC AND CHEMICAL CHANGES IN REGENERATING NEURONS—II

IN VITRO RATE OF INCORPORATION OF AMINO ACIDS INTO PROTEINS OF THE NERVE CELL PERIKARYON OF THE C.8 SPINAL GANGLION OF RABBIT

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THE eighth cervical (C.8) spinal ganglion of the growing and adult rabbit contains a constant number of nerve cell bodies (MIANI, unpublished). It is therefore possible to refer the rate of turnover of components of the perikaryon to an anatomical (nerve cell body count), instead of to a gravimetric standard. It is also known from the work of NAGEOTTE (1907) and CAJAL (1910) that spinal ganglia survive for a long time *in vitro*. Well controlled measurements of cell metabolism can therefore be made under reproducible conditions. These advantages justify the choice of the C.8 spinal ganglion for studies *in vitro* of the protein metabolism of the nerve cell perikaryon.

The present investigation was designed to study the rate of incorporation of free glycine, valine and threonine into ganglion cell proteins both at different ages and during regeneration of peripheral processes. It was hoped that information could be gained thereby about the relation of the protein metabolism of the perikaryon to the growth of peripheral processes.

MATERIAL AND METHODS

Animal experiments and method of operation. Altogether, 430 adult male rabbits of 1·8–2·5 kg weight (approx. 90 days old) and 88 growing male rabbits (6, 15 and 30 days old) were used for the experiments. It is known that, after emerging from the intervertebral foramen, each spinal nerve splits into a small posterior (dorsal) and an anterior (ventral) primary ramus (Fig. 1), each containing peripheral processes of the unipolar cells of the metameric spinal ganglion. In our experiments the anterior primary rami of the seventh and eighth cervical and first thoracic nerves of the right side were exposed under novocain anaesthesia, by dissecting the pectoral muscles. The anastomosis between the sixth and seventh cervical nerves (1), the seventh primary ramus (2) and the nerve trunk resulting from the eighth cervical and the first thoracic primary rami (3) (see Fig. 1) were crushed by forceps cooled to -70°. Pressure injury of the right cervical inferior sympathetic ganglion was also performed. In this manner the peripheral processes of the visceral sensory nerve cells of the C.8 and T.1 spinal ganglia, conveyed in the rabbit also through the grey rami comunicantes (STÖHR 1957), were destroyed. Seven hours latter, the right C.7, C.8, T.1 and sometimes the C.6 posterior primary rami of the same animal were exposed by dissecting the skin and the subcutaneous layer along the middle line of the nape. The nerves were crushed with cold forceps. By these operations the peripheral processes of the unipolar cells of the C.7 and C.8 spinal ganglia were severed, with the exception for each ganglion of those contained in the small and irregular meningeal and long thoracic branches.

Survival technique. At different intervals after pressure injury of nerves (from 2 to 78 days), the

animals were killed by bleeding; as soon as possible the cervical spinal cord and its posterior roots were exposed by cutting the laminae. The right C.8 ganglion, with 3 mm lengths of posterior root and spinal nerve were excised and after a quick washing in Krebs-bicarbonate solution they were transferred immediately to 2 ml of KREBS and HENSELEIT (1932) solution (pH 7·3–7·4 after equilibration with 5% CO_2 and 95% O_2), containing 200 mg per cent of glucose, 4·33 mg per cent of [^{14}C] glycine (sp. activity: 21·6 μc/mg), 1·46 mg per cent of L-[^{14}C]threonine (sp. activity: 32·9 μc/mg) and 988 μg per cent of L-[^{14}C]valine (sp. activity: 55·7 μc/mg). The preparation was placed in a pyrex-glass cylinder (2 × 7 cm) fitted with a two-hole rubber stopper: through the inlet tube there was a constant flow of moistened gas mixture containing 95% O_2 and 5% CO_2; the outlet tube was connected to a bubble-counter (BORSOOK and DUBNOFF, 1940). The rate of flow of the gas mixture through the reaction vessel was about one bubble per second.

During survival experiments the vessel was mounted on an arm of a Warburg apparatus for stirring and the lower half was immersed in water maintained at 37°.

Preparation and hydrolysis of the protein fraction of the spinal ganglion. At the end of the experiment the ganglion was quickly washed in fresh Krebs-bicarbonate solution and frozen in dry ice; the posterior root and the spinal nerve were severed with a razor blade and the spinal ganglion was placed in a glass-stoppered centrifuge tube (12 × 1·5 cm). All subsequent operations, including the coupling with fluorodinitrobenzene of the amino groups in the protein hydrolysate, were performed in this vessel.

The acid-soluble compounds, nucleic acids and ether-soluble compounds of the ganglion were extracted according to LAJTHA, FURST, GERSTEIN and WAELSCH (1957). The protein residue was hydrolysed in 0·5 ml of 6 N-HCl at 115° for 18 hr. For this stage the reaction vessel was put in a larger test tube containing 3 ml of 6 N-HCl, which was sealed before immersion in an oil bath at 115°. Excess HCl in the hydrolysate was removed by evaporation to dryness at 50° in an air stream.

Analysis of threonine, valine and glycine in the protein hydrolysate from the ganglion. For the quantitative analysis of threonine, valine and glycine Sanger's fluorodinitrobenzene method was used, as described by FRAENKEL-CONRAT, HARRIS and LEVY (1955). The hydrolysate was dissolved in 3 ml of an aqueous solution of $NaHCO_3$ (1% w/v), and to this was added 1·5 ml of an alcoholic solution of 1-fluoro-2:4-dinitrobenzene (FDNB)* (10%, w/v). The mixture was shaken for 2 hr at room temperature. Excess FDNB and ethanol were extracted with ether, the aqueous solution was acidified with 6 N-HCl, and the DNP-amino acids were extracted three times into ether (1 × 5, 3, 1 ml). The combined ether extracts in a round-bottomed flask were evaporated to dryness at 40° and excess dinitrophenol was sublimated according to MILLS (1952) (3 hr at 50°). The residue was quantitatively transferred to chromatography paper (Whatman No. 1) with small portions of acetone.

In order to separate all the ether-soluble DNP-amino acids the solvent system used in the first dimension was toluene–chloroethanol–piridine–aqueous ammonia (BISERTE and OSTEUX, 1951), and in the second was concentrated (1·5 M) phosphate buffer (pH 6) according to FRAENKEL-CONRAT *et al.* (1955). The yellow spots of DNP-glycine, DNP-valine and DNP-threonine were cut out of the chromatography paper with a razor blade about 4 mm beyond their visible periphery, and a blank of similar size was cut from the adjacent paper. Each of the spots and the blank were eluated with 1% $NaHCO_3$, taken up to 5 ml in volume and the optical density at 360 mμ (DNP-threonine and DNP-valine) and at 361 mμ (DNP-glycine) were read in the Beckman model DU Spectrophotometer. Whenever possible, the above procedure was performed in the dark. The optical density readings at E_{360} or E_{361} were converted to μg of amino acid using the curves in Fig. 2 which had been obtained by the addition of known amounts of glycine (Merck), L-threonine (Hoffmann-La Roche) and L-valine (Hoffmann-La Roche) to the protein hydrolysate in the above procedure. There was a linear relationship between the concentration of each amino acid and optical density of its DNP derivative over a wide range of amino acid concentration.

Determination of radioactivity and calculation of the flux of free threonine, valine and glycine from the non-protein nitrogen fraction of the C.8 spinal ganglion into ganglion cell proteins. After spectrophotometric measurements, the DNP-amino acid solution was transferred quantitatively to a planchet and brought to dryness under an infra-red lamp before radioactivity measurements. Radioactivity of all samples was determined in a gas-flow counter provided with an anti-coincidence shield. The background activity of the counter was only 2·2 ± 0·1 counts/min.

* Abbreviations used: 1-fluoro-2:4-dinitrobenzene, FDNB; dinitrophenol, DNP; non-protein nitrogen, NPN.

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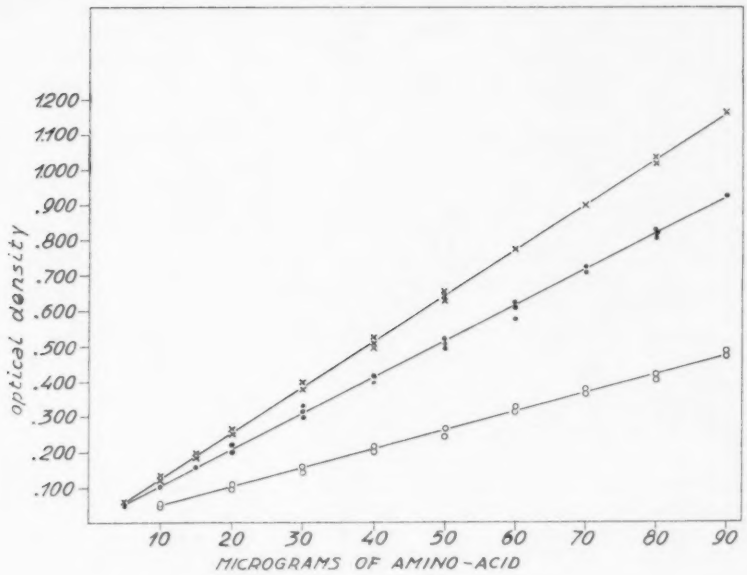

FIG. 2.—Correlation between glycine (×), threonine (●) and valine (○) concentration and optical density of their DNP-derivatives at E_{360} (threonine and valine) and at E_{361} (glycine).

If A is the total amount of an individual non-bound labelled amino acid in the amino acid pool at constant level, and B_1 and B_2 are the total amounts per organ of the same labelled amino acid bound to protein at times t_1 and t_2, the coefficient of specific velocity (K) can be calculated according to the familiar formula:

$$K(\text{sec})^{-1} = \frac{B_2 - B_1}{A(t_2 - t_1)}.$$

This calculation assumes a first-order reaction.

Total amount of labelled compounds in the non-protein nitrogen (NPN) fraction of the C.8 spinal ganglion. At different intervals after survival in Krebs-bicarbonate solution, containing glucose (200 mg/100 ml) and [^{14}C]glycine (4·33 mg/100 ml), valine (988 μg/100 ml), or [^{14}C]threonine. (1·46 mg/100 ml), the spinal ganglion was quickly immersed three times in a fresh physiological solution at room temperature, dried by filter paper and frozen in dry ice. The posterior root and the spinal nerve were severed with a razor blade, the organ was homogenized in 9 vols of cold 0·02 N-HCl and the suspension was centrifuged. The residue was homogenized in 0·02 N-HCl, separated by centrifugation, and the procedure repeated. Excess HCl in the combined protein-free supernatant solutions was removed by evaporation to dryness at 35° in an air stream. The residue was taken up in water, transferred quantitatively to a planchet and brought to dryness under an infra-red lamp before measuring the radioactivity in the gas-flow counter.

In order to determine the rate of movement of labelled compounds from the NPN fraction of the organ into the Krebs-bicarbonate solution, a set of organs, after survival for 30 min in bathing solution containing glucose and [^{14}C]glycine, [^{14}C]valine, or [^{14}C]threonine (concentrations as above), were transferred to fresh Krebs solution containing glucose and the respective unlabelled amino acid. After from 5 to 15 min in the non-radioactive solution, the organ was quickly immersed three times in physiological solution and the NPN fraction was extracted according to the procedure given above. The flux of labelled amino acids from the NPN fraction of the C.8 spinal ganglion was determined in adult and growing organs, as well as in regenerating ones; no significant differences were observed.

Localization of radioactivity in the NPN fraction. The combined acid-soluble fraction of several spinal ganglia, maintained for 75 or 105 min in Krebs solution (glucose and the experimental amino acid), were brought to dryness at 40° in an air stream. The residue was taken up in water, transferred to Whatman No. 1 filter paper and subjected to two-dimensional chromatography (Fig. 3).

Histological localization of labelled 'protein-bound' amino acids. Historadioautographs of spinal ganglia of growing and adult animals as well as of regenerating organs (2, 10, 18, 27 and 29 days after the nerve crush) were prepared by the melted emulsion technique (MIANI, 1956). Survival experiments with [^{14}C]glycine and [^{14}C]threonine (Fig. 4–7) lasted 20 and 75 min.

To determine the relative isotopic concentration in the individual nerve cell body and in the associated glial cells, the grain counting method was employed. As regards nerve cell bodies, only the grains visible by oil-immersed objective in a focal plane directly above a unipolar cell were counted; the grains visible at higher levels and those distant from the source of radiations were not computed. The background count was determined per unit area in a single plane of 'free space' made by microdissection in a region containing cells: this was subtracted from the total number of grains counted over the specimen.

The total number of grains visible in a focal plane directly above a glial cell was also counted, the underlying assumption here being that a satellite cell is wholly contained in sections 5 μ thick. Care was taken to choose glial cells associated with the cross-sectional area of the unipolar cell counted in radioactivity measurements. Preliminary analyses of 1000 gliocytes of adult ganglia, maintained for 20 and 75 min in Krebs-bicarbonate solution containing L-[^{14}C]threonine, have given $1{\cdot}2 \pm 0{\cdot}3$ and $2{\cdot}3 \pm 0{\cdot}2$ grains per cell respectively. These mean values were not affected by the age of the rabbit, nor by the chromatolytic stages of the nerve cells. There is a direct proportionality between the surface area of a unipolar cell body and the number of its satellite cells (MIANI, unpublished). The approximate total number of grains in glial cells encapsulating a nerve cell body are given in Table 1.

TABLE 1.—RELATIVE ISOTOPIC CONCENTRATION IN INDIVIDUAL NERVE CELL BODY AND IN GLIAL CELLS CORRELATED WITH SURFACE AREA

The C.8 spinal ganglia were maintained for 75 min in Krebs-bicarbonate solution containing [^{14}C]threonine before preparation of historadioautographs

100 day-old rabbit			13 day-old rabbit		
Nerve cell body volume (μ^3)	$N\frac{1}{2}$*	$n\frac{1}{2}$†	Nerve cell body volume (μ^3)	$N\frac{1}{2}$*	$n\frac{1}{2}$†
3,195 (d)§	178	25	2,793 (d)§	205	23
2,450 (d)	235	22	3,145 (d)	302	25
3,428 (d)	192	28	2,730 (d)	235	23
3,024 (d)	302	25	2,930 (d)	278	25
9,297 (l)	1836	51	7,107 (l)	710	41
10,845 (l)	651	55	7,827 (l)	690	46
10,490 (l)	254	55	6,436 (l)	398	39
12,429 (l)	707	62	9,012 (l)	1008	48
12,094 (l)	995	60	8,740 (d)	1490	48
12,094 (l)	1268	60	7,980 (d)	1850	46
39,367 (l)	1962	131	13,757 (l)	2540	66
90,825 (l)	2098	228	14,163 (l)	1985	69
82,380 (l)	2035	214	14,750 (l)	2302	71
49,540 (l)	2192	154	16,350 (l)	2183	76

* Half the number of reduced silver grains per nerve cell body.

† Half the number of reduced silver grains in glial cells encapsulating the nerve cell body.

§ In parentheses: d = 'dark' nerve cell body, l = 'light' nerve cell body.

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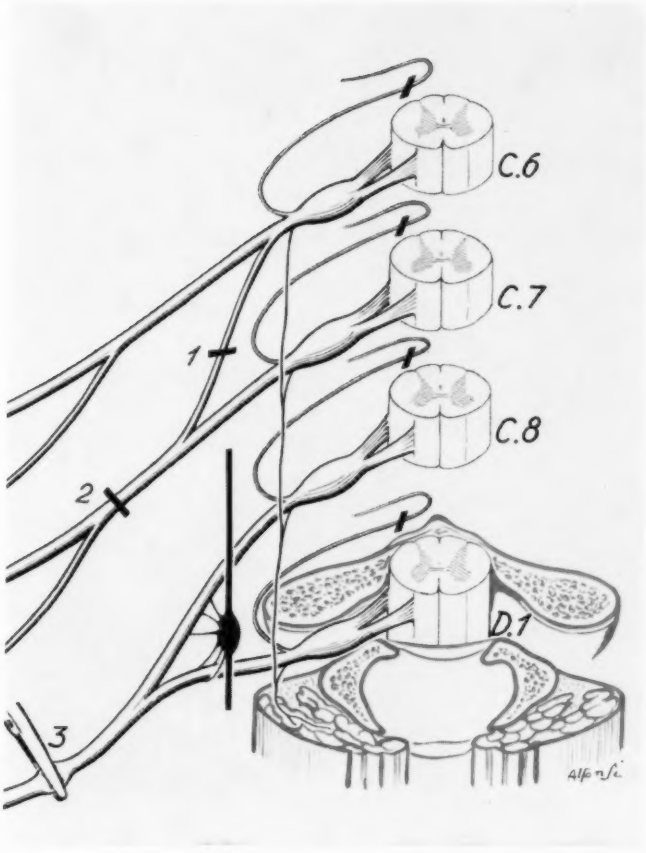

FIG. 1.—Schematic drawing of C.6, C.7, C.8 and D.1 spinal nerves of rabbit. The large lines on the nerves indicate the place of the pressure injury. Rami comunicantes unite the cervical inferior sympathetic ganglion and the eighth cervical and first thoracic primary anteriorrami.

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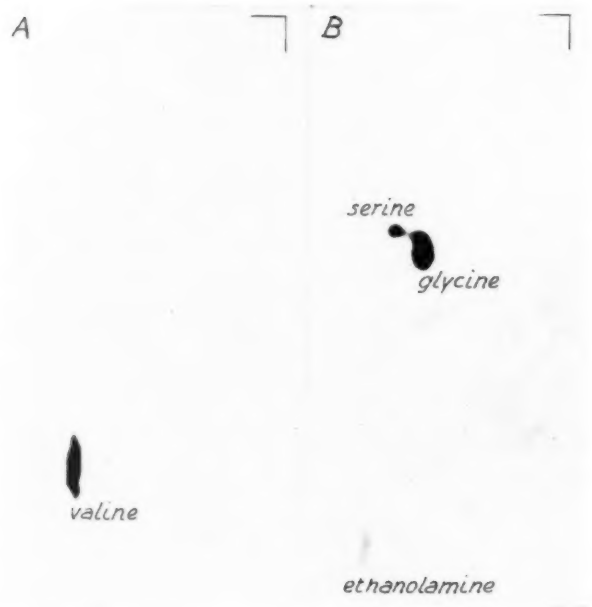

FIG. 3.—Radiochromatograms of acid-soluble compounds of C.8 spinal ganglion of adult rabbit incubated for 105 min with ^{14}C-labelled valine (A) and glycine (B). (Two-dimensional chromatograms: ↓ phenol/water; ← 'collidine', DENT, 1948).

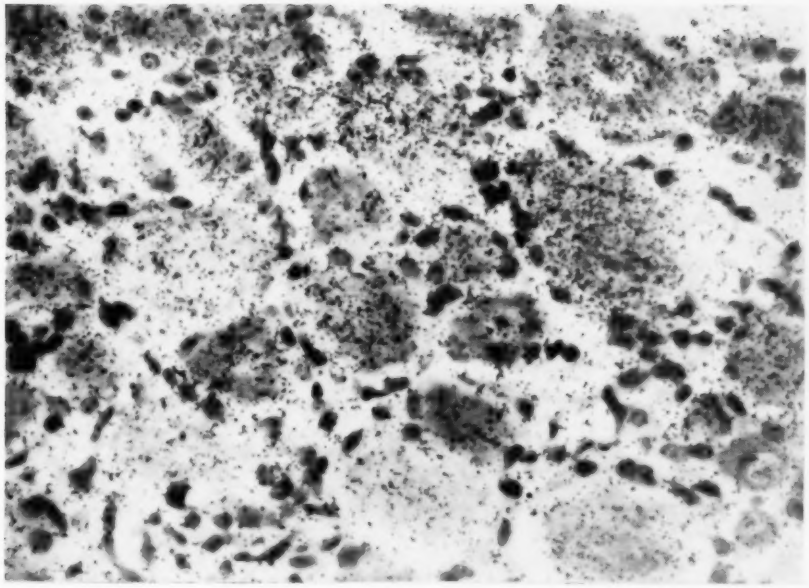

FIG. 4.—Radioautograph of the outer zone of a C.8 spinal ganglion of adult rabbit; the organ has been maintained for 75 min in Krebs-bicarbonate solution containing [^{14}C]threonine. The 'dark' nerve cell bodies exhibit a much heavier blackening than the adjacent 'light' ones. Gallocyanin-chromalum, ×520. Exposure: 4 months.

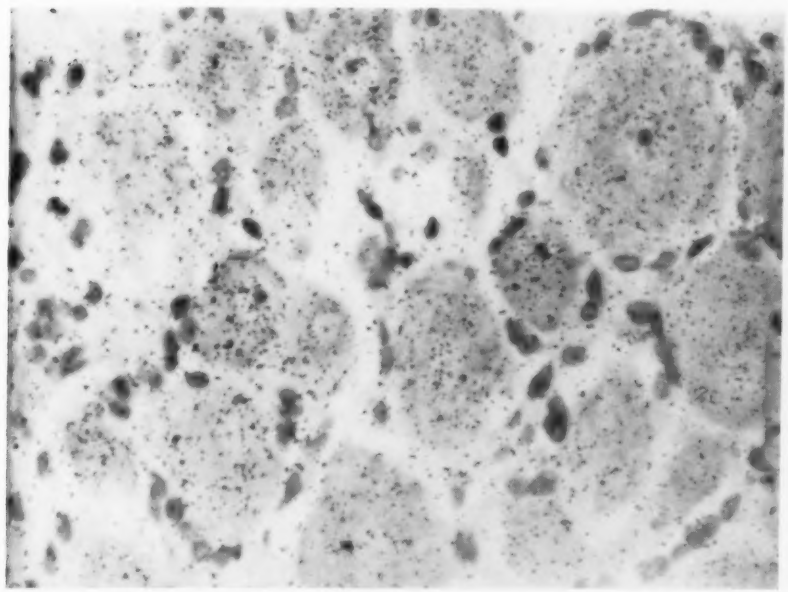

FIG. 5.—Radioautograph of the central zone of a C.8 spinal ganglion, one week after spinal nerve crush; survival for 20 min in Krebs-bicarbonate solution containing [^{14}C]threonine. The reduced silver grains are preferentially deposited over the nerve cell bodies. Gallocyanin-chromalum, ×520. Exposure: 4 months.

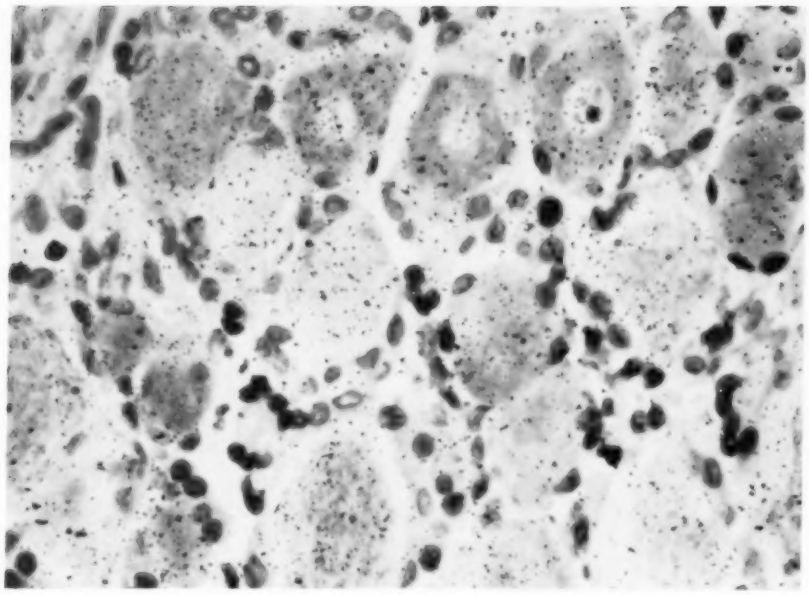

FIG. 6.—Radioautograph of the outer zone of the spinal ganglion of Fig. 5. There is no visible difference in the silver grain concentration between this region of the spinal ganglion and the central one, ×580.

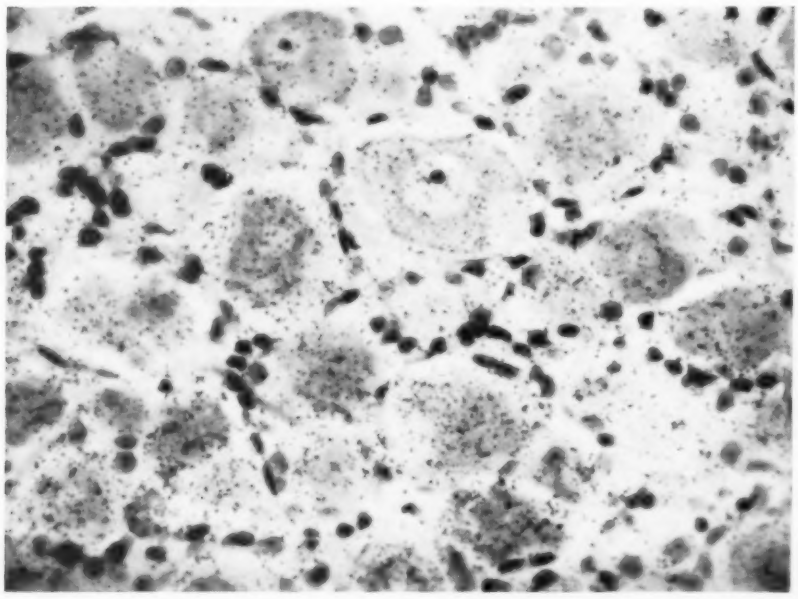

FIG. 7.—Radioautograph of the central zone of a C.8 spinal ganglion, one week after spinal nerve crush; survival for 30 min in Krebs-bicarbonate solution containing [^{14}C]threonine. The glial cells are nearly free of radioactivity. Gallocyanin-chromalum, ×520. Exposure: 4 months.

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RESULTS

Exchange of free threonine, valine and glycine between Krebs-bicarbonate solution and the non-protein nitrogen fraction of the C.8 spinal ganglion. The flux of labelled threonine, valine and glycine from Krebs-bicarbonate solution into the NPN fraction of the ganglion was determined for each amino acid. The radioactivity per organ increased quickly for 15–20 min (Fig. 8, left). After this the amount of free [^{14}C]threonine and [^{14}C]valine remained at a constant level; on the other hand, the free [^{14}C]glycine increased slowly for 75 min or longer.

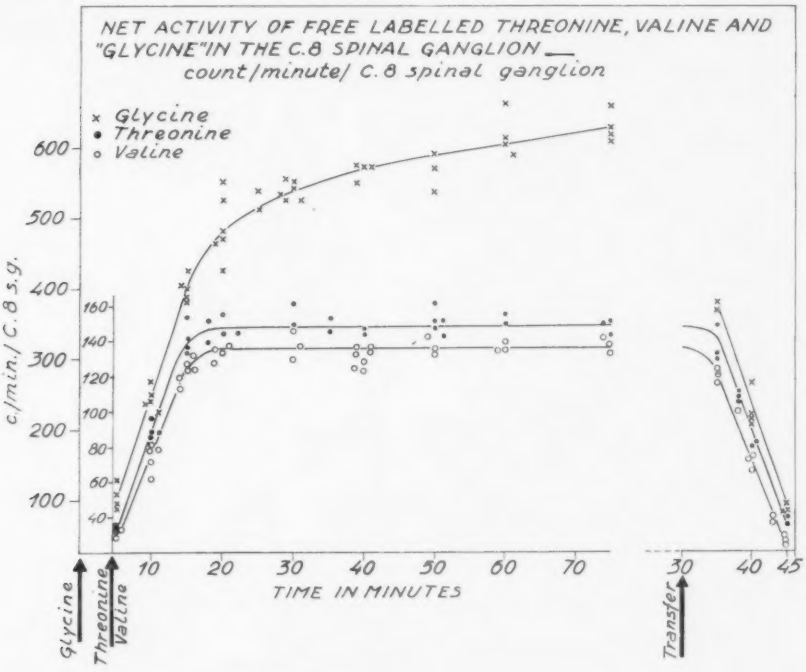

FIG. 8.—Total amount of the free [^{14}C]valine, [^{14}C]threonine and [^{14}C]glycine in the amino acid pool of the right C.8 spinal ganglion of adult rabbit as a function of survival time in the Krebs-bicarbonate solution containing labelled valine, threonine or glycine. To the right, the rate of disappearance of experimental amino acids from the amino acid pool of the right C.8 spinal ganglion of an adult animal. The transfer of the spinal ganglion from labelled to unlabelled Krebs-solution is indicated by an arrow.

The movement of free threonine, valine and glycine from the non-protein nitrogen fraction of the organ into Krebs-bicarbonate solution (Fig. 8, right) and *vice versa* (Fig. 8, left) occurred with the same velocity. Therefore, the exchange of the free experimental amino acids between the two compartments reached equilibrium within 15–20 min. After this, since the negligible amount of free amino acids in the ganglion cannot change the amino acid concentration of the Krebs solution, it is reasonable to assume that the value for the specific activity of each experimental amino acid in the amino acid pool of the organ agrees exactly with that of the bathing solution. However, direct evidence for this assumption has not yet been obtained

TABLE 2.—COEFFICIENT OF SPECIFIC VELOCITY OF FLUX OF THREONINE, VALINE AND GLYCINE FROM THE AMINO ACID POOL OF THE C.8 SPINAL GANGLION INTO GANGLION-CELL PROTEINS; AND THE TOTAL AMOUNT PER ORGAN OF PROTEIN-BOUND AMINO ACIDS DURING REGENERATION OF PERIPHERAL PROCESSES AND IN NON-OPERATED GROWING RABBIT

Experimental condition	Coefficient of specific velocity $K(\times 10^{-5}\ sec^{-1})$						Total amount of protein-bound amino acids per C.8 spinal ganglion $\mu g \pm$ S.D.		
Operated (days after)	Threonine	%†	Valine	%†	Glycine‡	%†	Threonine	Valine	Glycine
2	4·89	−9·6	5·35	−5·4	1·66	0·0	18·0 ± 2·9 (30)*	95·3 ± 10·7 (30)	19·1 ± 2·9 (30)
7	6·44	+19·0	6·51	+15·0	2·25	+35·5	22·6 ± 4·7 (28)	131·2 ± 11·3 (28)	31·4 ± 3·5 (28)
17	9·01	+66·5	7·64	+34·9	2·70	+62·6	30·4 ± 3·2 (32)	151·0 ± 30·4 (32)	34·2 ± 3·8 (32)
18	9·01	+66·5	7·64	+30·0	2·70	+62·6	30·2 ± 3·9 (27)	142·5 ± 12·5 (27)	34·9 ± 5·2 (27)
27	8·75	+61·7	7·36	+24·9	2·70		29·3 ± 3·6 (28)	139·0 ± 8·8 (28)	32·1 ± 3·1 (28)
40	8·50	+57·1	7·07	+19·0	2·25		25·9 ± 3·6 (28)	120·5 ± 7·9 (28)	29·7 ± 4·2 (28)
66	7·40	+36·7	6·79	+10·5	2·25	+35·5	27·5 ± 3·5 (32)	122·3 ± 10·2 (32)	52·0 ± 18·5 (32)
78	5·15	−4·8	6·71		1·94	+16·8	20·0 ± 2·8 (30)	109·1 ± 9·3 (30)	22·9 ± 4·1 (30)
Non-operated (days old)									
6	7·34	+35·6	7·00	+23·6	2·28	+37·3	6·7 ± 1·7 (26)	46·9 ± 4·4 (26)	10·5 ± 0·1 (26)
15	7·15	+32·1	6·80	+20·1	2·24	+34·9	9·3 ± 2·1 (33)	44·0 ± 3·7 (33)	11·4 ± 0·1 (33)
33	6·18	+14·2	6·10	+7·7	1·96	+18·0	12·1 ± 2·0 (29)	72·9 ± 4·8 (29)	17·3 ± 1·8 (29)
~90 (adult)	5·41		5·66		1·66		17·9 ± 1·2 (31)	101·0 ± 6·5 (31)	20·3 ± 1·2 (31)

* Number of spinal ganglia in parentheses.
† Percentage deviation from the coefficient of specific velocity of threonine, valine and glycine in non-operated C.8 spinal ganglion of adult animal (~90 days old).
‡ Corrected coefficient of specific velocity on the basis of a constant level of [^{14}C]glycine in the amino acid pool of C.8 spinal ganglion of 567 counts/min.

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because the total amount of the experimental amino acids in the spinal ganglion is too small.

During short-term experiments *in vitro*, [^{14}C]threonine and [^{14}C]valine of the amino acid pool of the organ were not converted into other compounds visible in a radioautograph (Fig. 4). Therefore the points for threonine and valine plotted in Fig. 8 express the net amount per organ of each amino acid. Glycine behaved in a different way: after survival for 75 or 105 min, the radioactivity in the NPN

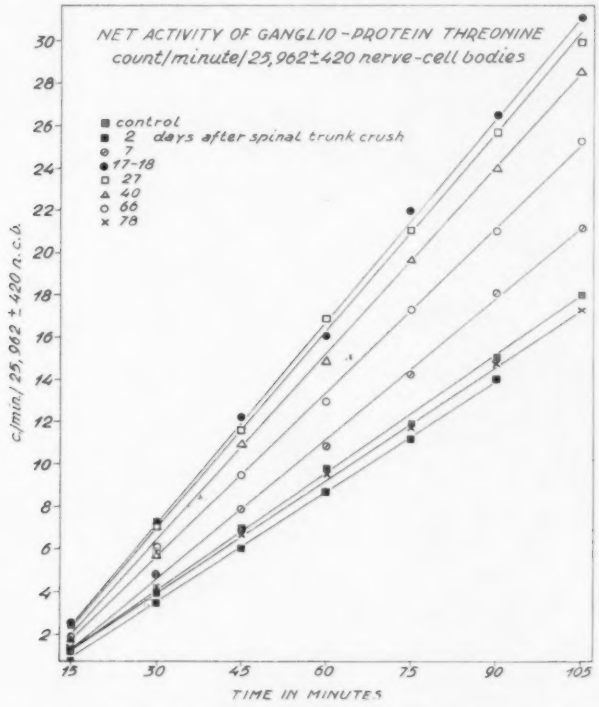

FIG. 9.—Net activity of protein-bound threonine of the right C.8 spinal ganglion of adult rabbit at different intervals after spinal nerve crush; survival experiments in Krebs-bicarbonate solution containing [^{14}C]threonine, [^{14}C]valine and [^{14}C]glycine. Each point is the mean value of 4 experiments with the exception of the sign ● (17, 18 days after nerve crush) which is the mean value of 8 experiments.

fraction of the ganglion was due primarily (about 90 per cent) to glycine itself, the other 10 per cent was due to its metabolic products (serine, ethanolamine) (Fig. 3).

Rate of incorporation of threonine, valine and glycine into proteins of the nerve cell bodies of the C.8 spinal ganglion during regeneration of peripheral processes. The flux of labelled threonine, valine and glycine from the amino acid pool of the ganglion into proteins of 25,692 $\pm$ 420 nerve cell bodies of the organ had a uniform velocity (Figs. 9–11), both in regenerating and non-operated adult neurons. The coefficient of velocity (K) of this flux is given in Table 2. Two days after spinal nerve crush the specific velocity of the threonine and valine fell by 9·6 per cent and 5·4 per cent respectively, as compared with control values; on the other hand, glycine did not

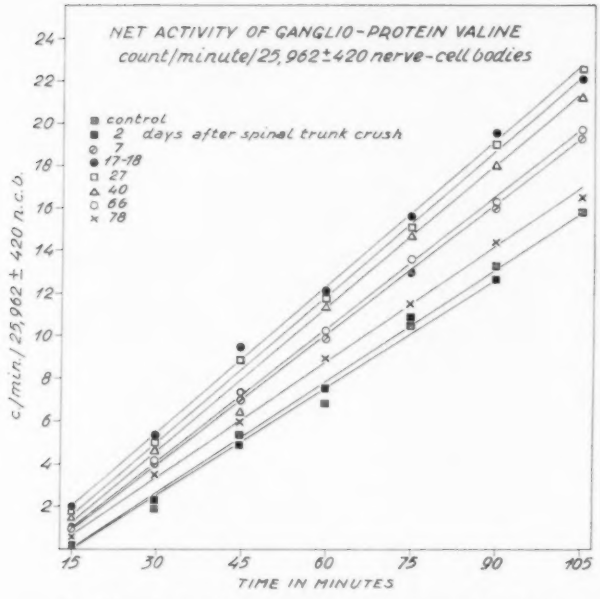

FIG. 10.—Net activity of protein-bound valine of the right C.8 spinal ganglion of adult rabbit at different intervals after spinal nerve crush; survival experiments in Krebs-bicarbonate solution containing [^{14}C]threonine, [^{14}C]valine and [^{14}C]glycine. Each point is the mean value of 4 experiments with the exception of the sign ● (17, 18 days after nerve crush) which is the mean value of 8 experiments.

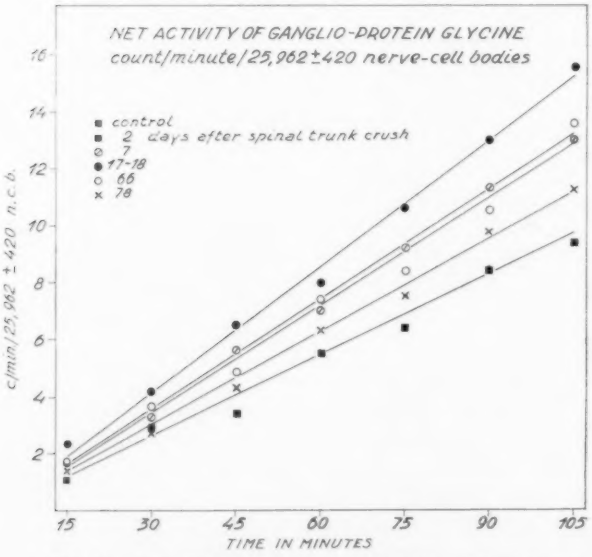

FIG. 11.—Net activity of protein-bound glycine of the right C.8 spinal ganglion of adult rabbit at different intervals after spinal nerve crush; survival experiments in Krebs-bicarbonate solution containing [^{14}C]threonine, [^{14}C]valine and [^{14}C]glycine. Each point is the mean value of 4 experiments with the exception of the signs ■ and ● which are the mean value of 8 experiments (■ = non-operated animals, and 2 days after nerve crush; ● = 17,18 days after nerve crush).

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change significantly. Subsequently a concordant increase in the value of K occurred for the experimental amino acids; the maximum was reached at 17 days, with an increase of 66·5 per cent for threonine, 34·9 per cent for valine and 62·6 per cent for glycine as compared with the controls. The coefficient of velocity for glycine, threonine and valine then went slowly back to the starting values.

Table 2 also summarizes the total amount of protein glycine, valine and threonine per organ during regeneration of peripheral processes as well as in control C.8 spinal ganglia. Although the validity of such values as a sign of pure neuronal changes may be questioned, our results parallel those obtained by BRATTGÅRD, EDSTRÖM and HYDÉN (1957) who studied the changes in RNA content and dry weight (protein and lipid fractions) per hypoglossal nerve cell body during nerve regeneration.

During short-time experiments *in vitro*, no significant removal of labelled ganglion cell proteins occurred, a fact confirmed by experiments reported below (see Table 3).

TABLE 3.—RATE OF DISAPPEARANCE OF PROTEIN-BOUND [^{14}C]-THREONINE FROM C.8 SPINAL GANGLION OF ADULT RABBIT

Experimental condition	No. ganglia	Counts/min per 25,962 ± 420 nerve cell body	Range
non-operated	7*	9·6 ± 1·6	8·3–12·0
	7†	9·6 ± 1·4	8·2–11·4
18 days after operation	7*	16·6 ± 2·1	14·5–18·5
	7†	16·4 ± 2·0	13·5–18·8

* C.8 spinal ganglia survived for 60 min in Krebs-bicarbonate solution containing [^{14}C]threonine.

† After 60 min in Krebs-bicarbonate solution containing [^{14}C]threonine the survival was continued in unlabelled Krebs's solution for a further 60 min.

Rate of incorporation of threonine, valine and glycine into proteins of the nerve cell bodies of the C.8 spinal ganglion of growing rabbits. As shown diagrammatically in Figs. 12 and 13, the labelled threonine and valine of the free amino acid pool of the ganglion were incorporated into proteins of a part of the ganglion containing 25,962 ± 420 nerve cell bodies at a rate which was a linear function of the experimental time. Glycine behaved in a similar way. The coefficient of velocity (K) of such a transfer for each age group is summarized in the lower part of Table 2. It is apparent that during the growth of the animal the flux of each experimental amino acid fell off exponentially. Owing to this, the velocities of incorporation of threonine, valine and glycine into ganglion cell proteins of a 90-day-old rabbit were reduced by 35·6 per cent, 23·6 per cent and 37·3 per cent respectively, as compared to the starting values for a 6-day-old animal.

Concomitant with the lowering of specific velocity of protein precursors, the total amount per organ of protein threonine, valine an glycine increased exponentially.

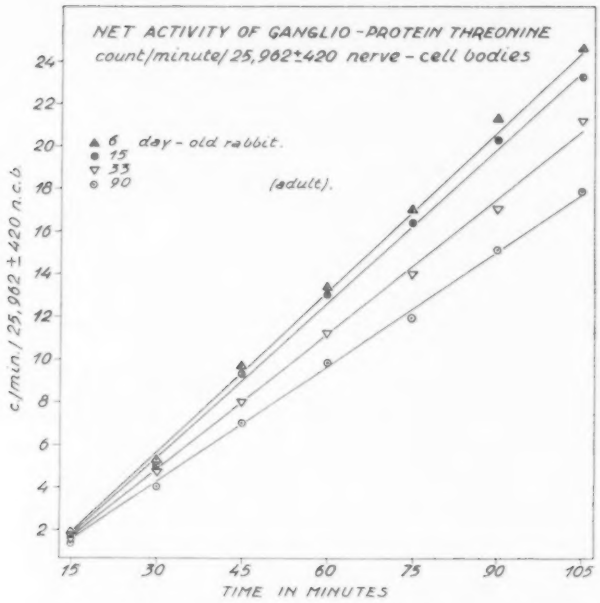

FIG. 12.—Net activity of protein-bound threonine of the right C.8 spinal ganglion of growing rabbit; survival experiments in Krebs-bicarbonate solution containing [^{14}C]threonine, [^{14}C]-valine and [^{14}C]glycine. Each point is the mean value of 4 experiments.

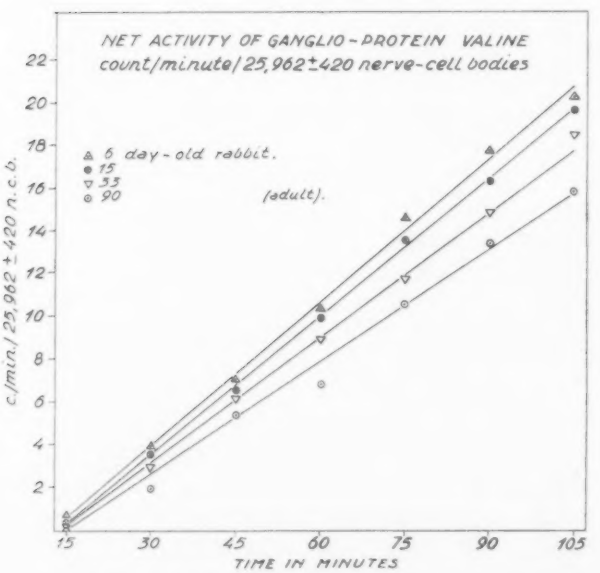

FIG. 13.—Net activity of protein-bound valine of the right C.8 spinal ganglion of growing rabbit; survival experiments in Krebs-bicarbonate solution containing [^{14}C]threonine, [^{14}C]-valine and [^{14}C]glycine. Each point is the mean value of 4 experiments.

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DISCUSSION

The quantitative histology of the C.8 spinal ganglion is a prerequisite for solving several problems related to the protein metabolism of the organ under different experimental conditions. Among the most important of these is the problem of reference standards for the expression of chemical and physical measurements on nervous tissue (POPE, 1955).

The mass unit of a protein amino acid from a ganglion is equivalent to a widely variable number of nerve cell bodies during the growth and regeneration of their peripheral processes. For example, 1 μg of protein-bound threonine was found in a part of organ containing 3874 $\pm$ 62 nerve cell bodies in a 6-day-old rabbit and 1450 $\pm$ 25 in a 90-day-old rabbit. It is clear that this precludes any accurate comparison of protein turnover in C.8 spinal ganglia differing in weight or volume. On the other hand, since the number of nerve cell bodies is constant, the flux of free labelled amino acids into proteins may be expressed in terms of the nerve cell body count. By this latter method, besides avoiding the necessity for weighing unipolar cells, we also avoid the need for measuring the concentration of connective tissue and glial cells. Clearly the exclusion of connective tissue in evaluating the rate of turnover of ganglion cell proteins is advantageous, since it is relatively inert metabolically, and therefore it lowers the specific activity of several amino acids in the ganglion cell proteins. In order partially to obviate this source of error, LAJTHA *et al.* (1957) have used [^{14}C]lysine, an amino acid that "occurs in at least as high concentrations in the proteins of parenchymatous tissue as in those of connective tissue". In the present investigation we were not obliged to choose any particular amino acids. Our reason for using glycine, valine and threonine was based on the fact that their DNP-derivatives are well separated on paper chromatograms.

It is of interest to note that similar results were obtained using these amino acids as indicators of protein metabolism, although glycine had the slowest rate of turnover (for comments see ARNSTEIN, 1954). These findings support the opinion (SIMPSON and VELICK, 1954) that the renewal of proteins takes place throughout the whole molecule, both in chromatolytic and non-operated growing nerve cell bodies.

It is reasonable to believe that protein fractions with widely different turnover rates occur in the spinal ganglia, as has been shown in the brain of the rat (CLOUET and RICHTER, 1957, 1959), cat (KRAVCHINSKY and SILICH, 1957) and monkey (FURST, LAJTHA and WAELSCH, 1958). One might also assume that in a single spinal ganglion there is a metabolic heterochronism associated with one or with a set of unipolar cells (Fig. 4–7); in fact, the suspected metabolic heterogeneity of neuronal proteins did not affect the uniform flux of amino acids into such proteins. Thus, during short-term experiments the coefficient of velocity of each experimental protein precursor may be calculated. From this point of view our results agree with those of GAITONDE and RICHTER (1956) who found that in short-term experiments the mean half-life of proteins did not differ greatly in growing and adult rat brain. Clearly, there is a simple relationship between our coefficient of velocity (K) and the half-life, $t_{\frac{1}{2}}$.

The data given in the present paper show that regeneration of the peripheral processes of unipolar cells is accompanied by a vigorous increase in protein synthesis in their nerve cell bodies, as compared with non-operated adult controls. Similar results have recently been reported by FISCHER, LODIN and KOLOUŠEK (1958) and by

BRATTGÅRD, HYDÉN and SJÖSTRAND (1958). The former authors studied radioautographically the distribution of [^{35}S]methionine in the facial nerve nucleus area, 13 days after nerve crush; the latter authors measured the incorporation of [^{14}C]lysine in a set hypoglossal cells, 2, 9, 21 and 207 days after pressure injury of the nerve. Nevertheless, it is difficult to compare the present findings with those of previous investigators in this field owing to the differences of material and methods employed.

According to experiments referred to elsewhere, MIANI (1961), the volume of an average unipolar cell of the C.8 spinal ganglion—the perikaryon and its peripheral and central axons—increases by about 1,100,000 μ^3 between a 6 ($\sim$300,000 μ^3) and a 90 ($\sim$1,400,000 μ^3) day-old rabbit. Crushing the brachial nerves, according to the method employed above, removes about 1,000,000 μ^3 of axoplasm from an average unipolar cell of the C.8 spinal ganglion of an adult animal. Therefore, the 6-day-old neuron and the crushed one are similar with regard to the rate of production of new protoplasm, to achieve the adolescent volume and to restore the lost axon, respectively. This, in spite of the considerable differences, linked the two groups of experiments (Table 2). During growth the velocity of flux of the experimental amino acids into proteins fell exponentially; at the same time the total amount of protein-bound experimental amino acids increased proportionally to the amino acid uptake. On the other hand, during regeneration of peripheral processes there seemed to be a direct relationship between the velocity of flux and the total amount of protein-bound amino acid in the ganglion. No critical evaluation of these results is possible until the rate of disappearance of protein from the nerve cell body is known. Following the distinct morphological observations of WEISS (1943, 1948), some experiments on the proximo-distal movement of protein along the axon—one form of protein loss from the nerve cell body—have been carried out using tagged compounds (SAMUELS *et al.*, 1951; WAELSCH, 1958, 1959; KOENIG, 1958; VERNE and DROZ, 1960). Further progress in this field may be obtained by a simple technique now being used in our laboratory (MIANI, 1960).

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SUMMARY

(1) The rate of incorporation of [^{14}C]threonine, [^{14}C]valine and [^{14}C]glycine into proteins of the C.8 spinal ganglion of the rabbit at different ages and during regeneration of peripheral processes of the unipolar cells has been studied *in vitro*.

(2) The flux of free amino acids from the amino acid pool of the organ into ganglion cell proteins has been referred to the number of the unipolar cells of the ganglion (our reasons for this are discussed) and the coefficient of specific velocity of such flux (K) has been calculated.

(3) The coefficients of specific velocity of changes in threonine, valine and glycine during regeneration of peripheral processes were observed for a period of 78 days. A concordant increase occurred for 17 days after spinal nerve crush; after this the coefficient of specific velocity for each experimental amino acid returned slowly to the starting values.

(4) During growth the value of K for the free amino acids fell exponentially. Because of this, the rates of incorporation of threonine, valine and glycine into ganglion cell proteins of a 90-day-old rabbit were reduced by 35·6 per cent, 23·6 per cent and 37·3 per cent respectively, as compared with the starting values for a 6-day-old animal.

(5) The total amount of protein-bound threonine, valine and glycine per organ varied in both chromatolitic and growing neurons.

Acknowledgement—The authors are indebted to Dr. DEREK RICHTER for the correction of the English.

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PURIFICATION OF URIDINE DIPHOSPHOGLUCOSE PYROPHOSPHORYLASE FROM HUMAN BRAIN*

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THE nucleotide UDPG‡ was first isolated from yeast (CAPUTTO *et al.*, 1950); and consists of a nucleoside linked by a pyrophosphate bridge. This nucleotide is synthesized from UTP and G-1-P by the enzyme uridine diphosphoglucose pyrophosphorylase. UDPG is one of the most important intermediate compounds in the metabolism of carbohydrates in brain.

Galactolipids are formed in brain by UDP-galactose donating the galactosyl group to a suitable lipid acceptor (BURTON, SODD and BRADY, 1958). The participation of UDPG in the interconversion of glucose to galactose is well established. Two processes are known: MAXWELL (1957) purified an enzyme uridine-diphosphogalactose-4-epimerase which is responsible for the formation of uridine diphosphogalactose from UDPG. Another enzyme Gal-1-P uridyl transferase (KALCKAR *et al.*, 1953) forms UDP-galactose from UDPG and Gal-1-P.

Recently it has been observed in this laboratory that UDPG is a precursor of glycogen in human brain (unpublished results). The enzyme for this glycogen synthesis is similar to UDPG-glycogen transglucosylase of LELOIR and CARDINI (1957).

Mucopolysaccharide formation also involves UDPG. DORFMAN and his coworkers (1958), have shown that the glucuronic acid moiety of uridine diphosphoglucuronic acid is directly incorporated into mucopolysaccharides. This nucleotide linked glucuronic acid is formed by the oxidation of UDPG by a specific DPN linked dehydrogenase. Work in this laboratory suggests that mucopolysaccharide is formed in brain. This has been demonstrated by the incorporation of radioactive sulphate into chondroitin sulphate during the early stages of rat brain development (GUHA *et al.*, 1960).

These three functions of UDPG emphasize the importance of uridine diphosphoglucose pyrophosphorylase. VILLAR-PALASI and LARNER (1960) purified this enzyme from rabbit skeletal muscle. The present paper describes the partial purification and properties of UDPG pyrophosphorylase from human brain.

MATERIALS AND METHODS

UDPG, UDP-N-Ac-Gm, TPN, UTP glucose-6-phosphate dehydrogenase, and galactose-adapted yeast were obtained from Sigma Chemical Company, U.S.A. $Na_2H^{32}PO_4$ was obtained from Atomic Energy Establishment, India. ^{32}P-labelled pyrophosphate was prepared by heating $Na_2H[^{32}P]O_4$ at 400° for 1 hr. Gal-1-P was prepared according to the method of ANDERSON, MAXWELL and BURTON

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† Junior Research Fellow, Council of Scientific and Industrial Research, Government of India.
‡ Abbreviations used: UDPG, uridine diphosphoglucose; UDP-N-Ac-Gm, uridine diphospho-N-acetyl glucosamine; UTP, uridine triphosphate; tris, 2-amino-2-hydroxymethylpropane-1:3-diol; G-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-phosphate; TPN, Triphosphopyridine nucleotide.

(1959), using galactokinase extracted from yeast. Gal-1-P transuridylase activity was measured according to the method of MAXWELL, KALCKAR and BURTON (1955). Inorganic pyrophosphatase was purified from yeast according to the method of HEPPEL and HILMOE (1951). Protein was estimated according to the method of WARBURG and CHRISTIAN (1941).

Assay. Pyrophosphorylase was assayed by measuring the disappearance of pyrophosphate in the presence of UDPG. This was possible as brain inorganic pyrophosphatase is inactive in the absence of cysteine (SEAL and BINKLEY, 1957). The assay system contained 30 μmoles of Tris pH 8·0, 2 μmoles of $MgCl_2$, 0·5 μmoles of UDPG, 1 μmole of $Na_4P_2O_7$ (adjusted to pH 8·0), and appropriately diluted enzyme in a final volume of 0·25 ml. This mixture was incubated at 37° for 30 min. The reaction was stopped by heating the incubation mixture to 100° for 1 min. After cooling, 50 μmoles of Tris buffer pH 7·4 and 0·25 μg of inorganic pyrophosphatase were added. The mixture was then incubated at 37° for 10 min. The inorganic phosphate formed was measured by the method of FISKE and SUBBAROW (1925). The assay gives a linear curve with respect to the concentration of pyrophosphorylase (Fig. 1). One unit of enzyme was expressed as the disappearance of 1 μmole of pyrophosphate in 30 min in the presence of UDPG under the assay condition.

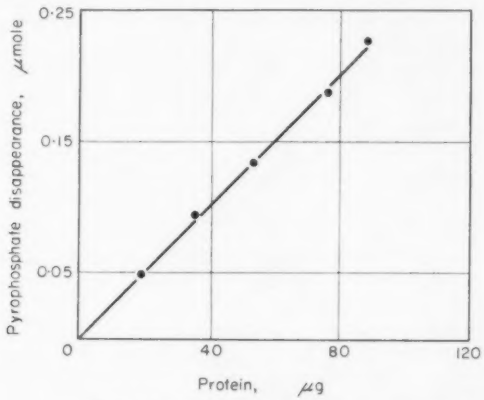

FIG. 1.—Relation of reaction rate to enzyme concentration. The assay conditions are as described in the text except for the amount of enzyme which is shown.

Purification of enzyme. Operations were carried out at 0°–5° unless otherwise mentioned. *Extraction.* Defatted frozen human brain (200 g) was homogenized for 2 min with 500 ml of 0·01 M-sodium pyrophosphate in a Waring blender. The homogenate was mechanically stirred for 30 min and centrifuged at 18,000 **g** for 30 min in a Servall centrifuge. The supernatant liquid (400 ml) was dialysed against 20 vol distilled water for 6 hr. The dialysed solution contained 3·86 g protein in 410 ml. *First calcium phosphate gel treatment.* 1025 ml of calcium phosphate gel (7·5 mg/ml) was added to the dialysed solution (gel to protein ratio was 2:1). The mixture was stirred for 15 min. The gel was collected by centrifuging at 6,000 **g** for 10 min, was washed with 200 ml 0·005 M-sodium pyrophosphate (pH 8·0), and then eluted with 250 ml of 0·01 M-pyrophosphate (pH 9·7). The gel eluate contained 1·6 g of protein in 246 ml. *First ammonium sulphate fractionation.* Gel eluate (246 ml) was brought to 35% ammonium sulphate saturation by adding, with constant stirring, 47·74 g of the salt. The mixture was centrifuged and the precipitate was discarded. The supernatant solution was brought to 60% ammonium sulphate saturation by adding 39·76 g of the salt. The precipitate was collected by centrifugation and was dissolved in 40 ml of 0·005 M-pyrophosphate (pH 8·0). The solution was then dialysed overnight against 50 vol of 0·005 M-Tris (pH 7·4). The dialysed solution contained 0·40 g protein in 45 ml. *Second gel treatment.* After adjusting the pH of the dialysed solution to 6·0 with M-acetic acid, 54 ml of the gel was added (gel to protein ratio was 1:1). The supernatant liquid, obtained after centrifugation, contained 0·110 g protein in 90 ml. *Second ammonium sulphate fractionation.* The gel supernatant liquid was immediately brought to an ammonium sulphate concentration of 45% saturation by adding 23·04 g of the salt. After centrifugation the

Table 1.—Purification of UDPG pyrophosphorylase from human brain

Purification stage	Volume (ml)	Total protein (mg)	Total units*	Activity (units/mg)
Extract	410	3860	17,630	4·56
Gel eluate	246	1600	17,466	10·84
First ammonium sulphate	45	400	14,625	36·11
Gel supernate	90	110	13,950	127·05
Second ammonium sulphate	6·5	40	6,338	155·50

* μmoles of pyrophosphate disappearing in 30 min.

precipitate was discarded and the concentration of ammonium sulphate in the supernatant solution was increased to 60% by adding an additional 8·28 g of the salt. The precipitate, collected by centrifugation, was dissolved in 0·005 M-pyrophosphate (pH 8·0). The solution contained 0·04 g protein in 6·5 ml. The purification procedure is summarized in Table 1. A 32-fold purification with an overall activity of 36 per cent of the original activity was achieved.

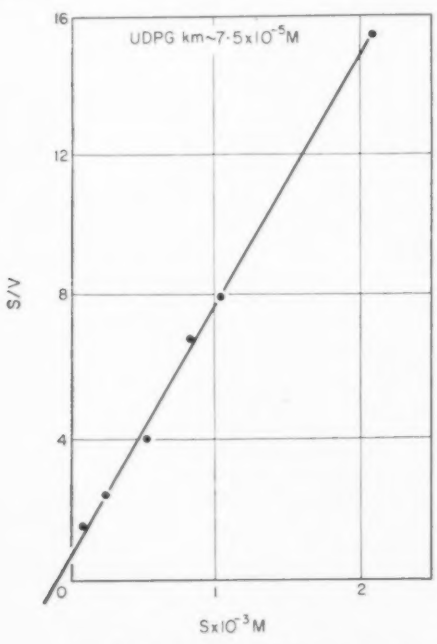

Fig. 2.—K_m value for UDPG. Assay conditions are described in the text. 6 μg of enzyme protein were used.

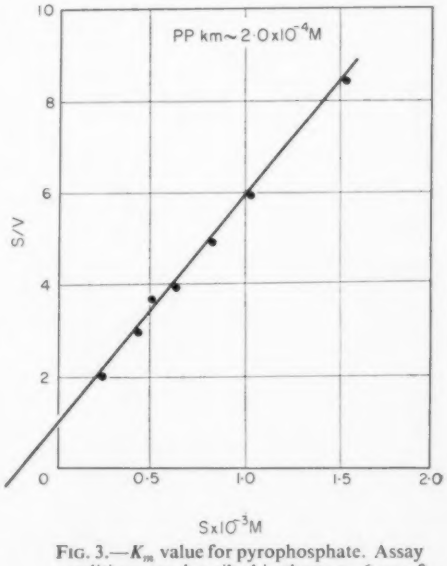

Fig. 3.—K_m value for pyrophosphate. Assay conditions are described in the text. 6 μg of enzyme protein were used.

Results and Discussion

The purified UDPG pyrophosphorylase was found to be free from Gal-1-P transuridylase and UDP-N-Ac-Gm pyrophosphorylase. The purified enzyme was stable for at least two months when stored at −15°. Cysteine or glutathione had no

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effect on the activity of the pyrophosphorylase. *p*-Chloromercuribenzoate at a concentration of 10^{-4}M inhibited the activity of this enzyme only to the extent of 50 per cent. These results demonstrated that the brain pyrophosphorylase had a low sensitivity to sulphydryl reagents.

The Michaelis constant for UDPG was $7{\cdot}5 \times 10^{-5}$M and for pyrophosphate was 2×10^{-4}M (Figs. 2 and 3). These values are slightly higher than those of the UDPG pyrophosphorylase from skeletal muscle as reported by VILLAR-PALASI and LARNER (1960).

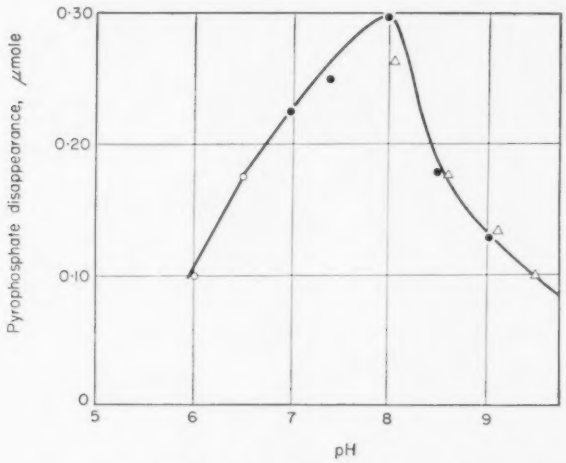

FIG. 4.—Effect of pH on the enzyme reaction. The conditions are described in the text. 6 μg of enzyme protein were used. Incubated for 30 min at 37°.
○—Tris–maleate buffer
●—Tris–HCl buffer
△—Glycine–NaOH buffer

The effect of pH on the activity is shown in Fig. 4. Enzyme activity has a sharp optimum pH at 8·0. This pH optimum is similar to that shown by mung bean enzyme (GINSBURG, 1958) but differs from that of muscle phosphorylase. This difference may be due to the longer incubation period employed in the present study.

The enzyme had an absolute and specific requirement for Mg^{++} for its activity. Other divalent metal ions, Mn^{++}, Zn^{++}, Ni^{++}, Co^{++} and Cu^{++}, were found to be ineffective in replacing Mg^{++}. Ca^{++} was also found to be ineffective in replacing Mg^{++} and, at a comparable concentration, it had no effect on the enzymic activity when Mg^{++} was present.

An optimal Mg^{++} concentration of 4 mM for the maximum enzyme activity was found when the concentration of pyrophosphate was 4 mM. At this concentration of pyrophosphate, increased concentration of Mg^{++} inhibited the pyrophosphorylase activity (Fig. 5). The inhibitory effect of Mg^{++} at a high concentration is consistent with the findings of VILLAR–PALASI and LARNER (1960).

G-1-P and Mg^{++} were needed for the pyrophosphate exchange with UTP (Table 2). Gal-1-P did not inhibit this exchange. A slight exchange in the presence of Gal-1-P may be due to G-1-P present in the Gal-1-P preparation.

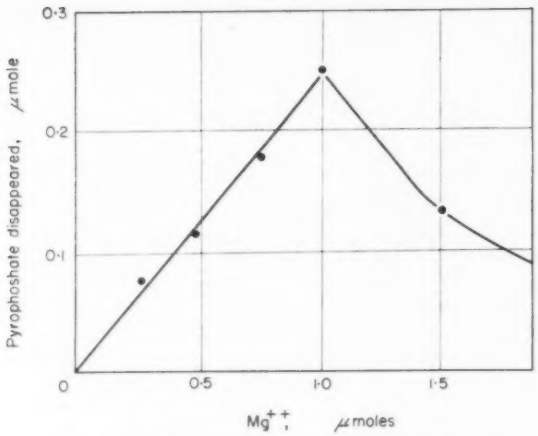

Fig. 5.—Effect of Mg^{++} on the enzyme reaction. The conditions are described in the text. 7 μg of enzyme protein was used.

Table 2.—UTP–pyrophosphate exchange

Components	Counts/100 sec
Complete	7230
Minus G-1-P	100
Minus Mg^{++}	115
Minus Enzyme	180
Gal-1-P (minus G-1-P)	485
Complete plus Gal-1-P	7445

The incubation was carried at 37° for 30 min in a total volume of 0·4 ml. The incubation mixture contained 50 μmoles Tris (pH 8·0), 2 μmoles $MgCl_2$, 0·5 μmole UTP, 1 μmole G-1-P, 1 μmole ^{32}P (46,900 counts/100 sec/mole) and 6 μg of the purified enzyme, 1 μmole of Gal-1-P was added as indicated in the table. The reaction was stopped by adding 0·2 ml of 10% perchloric acid in the cold. 100 mg of activated DARCO 60 was added to the deproteinised solution and the charcoal was washed. The nucleotide was eluted according to the method of Crane and Lipmann (1953) and counted.

SUMMARY

Uridine diphosphoglucose pyrophosphorylase has been purified 30-fold from human brain. The enzyme has an absolute and specific requirement for Mg^{++}. Enzyme activity is optimum at pH 8·0. K_m for UDPG is $7{\cdot}5 \times 10^{-5}$M and for pyrophosphate is 2×10^{-4}M.

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EFFECT OF CHLORPROMAZINE ON LEVELS OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE OF BRAIN

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CHLORPROMAZINE is a tranquilizing agent which is capable of modifying a large number of metabolic processes. The drug is able to inhibit oxidative metabolism of brain homogenates (BERNSOHN, NAMAJUSKA and COCHRANE, 1955; CENTURY and HORWITT, 1956; DECSI and MÉHES, 1957; MESSER, 1958), slices (FINKLESTEIN, SPENCER and RIDGEWAY, 1954; RAU *et al.*, 1956), and mitochondrial preparations (ABOOD, 1955). The increased oxygen consumption of cortical slices due to electrical stimulation or potassium stimulation is especially sensitive to the presence of the drug (MCILWAIN and GREENGARD, 1957; LINDAN, QUASTEL and SVED, 1957*a*). Chlorpromazine has been reported to inhibit several of the steps of the respiratory chain (BERGER, 1957), including DPNH-cytochrome *c* reductase (DAWKINS, JUDAH and REES, 1959*b*) and cytochrome oxidase (BERNSOHN *et al.*, 1955; ABOOD, 1955; BERNSOHN, NAMAJUSKA and BOSHES, 1956; DAWKINS, JUDAH and REES, 1959*a*). Several workers have demonstrated that the drug is capable of uncoupling oxidative phosphorylation, in some instances in concentrations which do not affect oxidative metabolism (CENTURY and HORWITT, 1956; DECSI and MÉHES, 1957; ABOOD, 1955; ANDREJEW *et al.*, 1956*a*). BERGER (1957) noted that oxidative phosphorylation in liver mitochondria was more sensitive to chlorpromazine than that in brain mitochondria, and concluded brain mitochondrial effects were relatively nonspecific. However, DAWKINS *et al.* (1959*a*), using a somewhat different system, found that brain mitochondria were quite sensitive to the uncoupling action of chlorpromazine. MESSER (1958) concluded that reduced phosphorylation due to chlorpromazine was secondary to the depression of oxidative metabolism.

Chlorpromazine is known to inhibit both aerobic and anaerobic glycolysis (BERNSOHN *et al.*, 1955; ANDREJEW and ROSENBERG, 1957). ATP-ases are inhibited by the drug (ABOOD, 1955; ANDREJEW *et al.*, 1956*b*; BERNSOHN *et al.*, 1956; MESSER, 1958). In addition the drug has been reported to inhibit cholinesterases (BERNSOHN *et al.*, 1956; ERDÖS, BAART, SHANOR and FOLDES, 1958), glutamine synthesis (MESSER, 1958), the incorporation of glycine into protein (LINDAN, QUASTEL and SVED, 1957*b*), D-amino acid oxidases (YAGI, NAGATSU and OZAWA, 1956; LASSLO and MEYER, 1959), and the turnover of brain phospholipid phosphorus (ANSELL and DOHMAN, 1956; RICHTER, 1957).

The vast number of biochemical processes affected by chlorpromazine renders the elucidation of the primary pharmacological action of the drug exceedingly difficult. The reactions listed, however, can be grouped into two large categories: (1) those which are believed to be primarily involved in oxidative phosphorylation and the

synthesis of ATP,* and (2) those which require the utilization of ATP. Inhibition of one or more of the reactions in the first group would be expected to result in interference with the phosphorylation of adenine nucleotides in brain and consequent limitation or reduction of the supply of available energy necessary for cerebral function. The second group of reactions, if inhibited, would lead to impairment in the utilization of ATP. Thus, if one or more of the first effects are responsible for the pharmacological action of chlorpromazine, one might expect to find reduced levels of labile phosphate compounds in brains of treated animals. If, on the other hand, the utilization of ATP were inhibited, either directly or indirectly, one might expect to find either normal or elevated levels of these compounds.

GRENELL, MENDELSON and MCELROY (1955) studied this problem, measuring ATP by the luciferase method. They found the levels of ATP in chlorpromazine-treated animals to be significantly higher in several regions of rat brain, including thalamus, hypothalamus, and cortex. Their results suggested some selective interference with ATP utilization by chlorpromazine. These workers, however, reported levels of ATP in both control and treated groups which ranged around 60–150 μg per g tissue, or about 0·1–0·3 μmole/g, levels considerably lower than those which have been reported by other investigators (MCILWAIN, 1959). In their studies, the rats were decapitated, the brains treated with boiling water, and ATP levels measured in these aqueous extracts. In subsequent work, using the decapitation procedure, but treating with perchloric acid, GRENELL, MAY, MENDELSON and MCELROY (1959) found slightly higher levels of ATP in rat brain, but were no longer able to detect a difference between control and chlorpromazine-treated rats. They concluded that chlorpromazine may in some way modify the distribution and binding of ATP *in vivo* and so render it less easy to extract from tissues. This alteration in binding might lead to interference with ATP utilization in specific metabolic systems, possibly the protein-synthesizing systems, since the chief binding alteration appeared to be in the microsomal fraction of cells.

It has been known for some time that the amounts of labile phosphate compounds which may be recovered from brain tissue are critically dependent on the method employed in killing the animal and fixing the tissue. KERR (1935) demonstrated that quick-freezing of intact brain with liquid air results in high recoveries of labile phosphate compounds. Prior anaesthetization increased recoveries even more. In view of this, we decided to evaluate the effect of chlorpromazine administration upon the brain content of adenine nucleotides and CP, using a quick-freezing procedure to minimize postmortem degradation of labile phosphate compounds.

METHODS

Rats, 52–135 g in weight, were given very large doses of chlorpromazine hydrochloride subcutaneously, ranging from 50 mg/kg body weight to as high as 800 mg/kg body weight. Animals given doses between 300–800 mg/kg were severely depressed, were somewhat ataxic, but were awake and responded to external stimuli. About 1 hr after the last dose, the animals were killed by immersion in liquid oxygen. Controls were injected in a similar manner with saline, and similarly killed. In some experiments, control and treated rats were killed by decapitation, the head being allowed to fall into liquid oxygen. The frozen brain was chiselled out, pulverised in a steel homogeniser, and treated with perchloric acid (WEINER, 1961). The extract was neutralised and AMP

* The abbreviations used are: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CP, creatine phosphate.

ADP and ATP were determined by WEINER's (1961) modifications of the method of KALCKAR (1947). Free and total creatine were determined by the procedure described by ENNOR and ROSENBERG (1952). CP was obtained by difference. Homogenates from control and chlorpromazine-treated animals were assayed for creatine phosphokinase, and their ability to liberate inorganic phosphate from ATP, ADP and AMP. After the chlorpromazine-induced depression was maintained for at least 30 min, the animal was killed by a blow on the head, and the brain was rapidly removed and homogenized in 0·2 M-Tris buffer, pH 7·6 containing 0·01 M-$MgCl_2$. Assays were performed on the brain homogenates and similarly prepared homogenates from control animals with AMP, ADP, ATP and CP as substrates (WEINER, 1961).

RESULTS

Immersion method. The levels of adenine nucleotides and CP in both control and chlorpromazine-treated animals are identical, except that the AMP level in brains of the treated group is slightly, but significantly ($P < 0{\cdot}05$), higher than in the controls (Table 1). There is considerable variation from animal to animal, and this variation is related to brain sample size. ATP and CP levels are inversely related to brain sample size, whereas AMP levels are directly related to the size of the brain sample. This is analogous to results reported previously (WEINER, 1961). The same relationship existed for both control and treated animals.

Decapitation method. When animals are killed by decapitation, there is a marked decrease in levels of ATP and CP in the brains of control rats, and a rise in AMP and ADP. In the control group, both ATP and CP are about one-half the values obtained by the immersion procedure. The AMP concentration is about twice as high as that observed for animals killed by immersion. Though the same trend is noted in the values from brains of chlorpromazine-treated rats who were subsequently decapitated, the changes are not nearly as great. The ATP and CP levels of the chlorpromazine-treated group are significantly higher than those of the control group, whereas the AMP is significantly lower (Table 2).

Enzyme studies. The activities of creatine phosphokinase and the enzymes involved in the dephosphorylation of adenine nucleotides in brains from chlorpromazine-treated animals are not significantly different from those of the controls. (Table 3).

DISCUSSION

The results presented here indicate that the inhibitory actions of chlorpromazine on either oxidative metabolism or oxidative phosphorylation are not related to the primary pharmacological action of the drug. The acid extraction of labile phosphate compounds is not altered by the prior administration of chlorpromazine (GRENELL *et al.*, 1959). The massive stimulation of decapitation, on the other hand, results in a lower rate of destruction of labile phosphate compounds in the chlorpromazine-treated group, as compared with controls. This suggests that chlorpromazine interferes with the utilization of ATP in the brain, either directly or indirectly through a depression of brain function, with the result that an equivalent external stimulus leads to less intense cerebral activity. The depression of ATP-ase by chlorpromazine *in vitro* (ABOOD, 1955; ANDREJEW *et al.*, 1956*b*; BERNSOHN *et al.*, 1956) is consistent with this hypothesis and could explain the *in vivo* nucleotide levels we have observed in the immersion and decapitation experiments. The pharmacological significance of these *in vitro* observations, however, is difficult to assess, since the addition of chlorpromazine in rather high concentrations is required for significant inhibition. In addition, chlorpromazine appears to be taken up from the medium into the tissue, effecting a

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TABLE 1.—BRAIN LABILE PHOSPHATE COMPOUNDS IN CONTROL AND CHLORPROMAZINE-TREATED ANIMALS. IMMERSION METHOD

	AMP μmole/g	ADP μmole/g	ATP μmole/g	CP μmole/g	Mean brain sample size (g)
Control (14)*	0·51	0·52	0·94	1·32	1·15
S.D.†	0·03	0·02	0·05	0·07	
Chlorpromazine-treated (15)	0·59‡	0·52	0·94	1·34	1·17
S.D.	0·02	0·03	0·06	0·08	

* Number of animals in each group in parentheses.
† S.D. = standard deviation of the mean.
‡ $P < 0·05$ that difference between chlorpromazine-treated group and control group is due to chance.

TABLE 2.—BRAIN LABILE PHOSPHATE COMPOUNDS IN CONTROL AND CHLORPROMAZINE-TREATED ANIMALS. DECAPITATION METHOD

	AMP μmole/g	ADP μmole/g	ATP μmole/g	CP μmole/g	Mean brain sample size (g)
Control (20)*	1·02	0·82	0·55	0·61	1·14
S.D.†	0·06	0·06	0·05	0·05	
Chlorpromazine (20)	0·81	0·79	0·73	0·85	1·29
S.D.	0·03	0·04	0·05	0·06	
P‡	<0·01	NSD§	<0·02	<0·01	

* Number of animals in each group in parentheses.
† S.D. = standard deviation of the mean.
‡ P = probability that differences between chlorpromazine-treated group and control group are due to chance.
§ NSD = no significant difference.

TABLE 3.—RATES OF BREAKDOWN OF LABILE PHOSPHATE COMPOUNDS BY BRAIN HOMOGENATES

Group	Substrate	μmole P liberated per g tissue per sec	μmole creatine liberated per g tissue per sec
Control (5)*	ATP	0·61 ± 0·06	—
Chlorpromazine (5)	ATP	0·60 ± 0·06	—
Control (3)	ATP–CP	0·80	0·65
Chlorpromazine (3)	ATP–CP	0·82	0·64
Control (3)	ADP–CP	0·56	1·17
Chlorpromazine (3)	ADP–CP	0·57	1·24
Control 4° (5)	ATP	0·10 ± 0·03	—
Chlorpromazine 4° (5)	ATP	0·13 ± 0·03	—

* Number of separate determinations of enzymic activity. Figures represent mean ± standard deviation of the mean.

further elevation of the tissue drug content (CHRISTENSEN and WASE, 1956; SALZMAN and BRODIE, 1956; MCILWAIN and GREENGARD, 1957). We have attempted to circumvent this difficulty by producing a pharmacological effect *in vivo* and then assaying the enzymic activity of the brain. No significant differences in the activities of enzymes involved in the catabolism of labile phosphate compounds between brains from drug-treated and control animals were detected. These negative results are also difficult to interpret because, although chlorpromazine is bound to tissue constituents, its relatively brief duration of action *in vivo* implies a reversibility of pharmacological action and suggests the possibility that dilution of the brain homogenate may have led to the loss of an inhibitory effect.

The higher levels of labile phosphate compounds in brains of chlorpromazine-treated rats observed by GRENELL *et al.* (1955) and DECSI and MÉHES (1957) appear to result from the more rapid deterioration of these compounds in control animals during the killing of the animals and the manipulation of the brain tissue before denaturation of the brain proteins. These differences are therefore not directly attributable to the *in vivo* action of the drug, although they may be related to the mechanism by which the drug exerts its depressant effect.

The brain levels of ATP and CP appear somewhat lower, and the level of AMP appears somewhat higher, than those reported previously (WEINER, 1961). This may be explained by the observation that the mean brain sample size in the present study was considerably larger than that in the previous study. If the volumes of extracting fluid are below an optimal level, the larger the brain sample size the less complete will be the extraction of CP and ATP, and the more likely some enzymic degradation of ATP and ADP will occur. If these factors are taken into account in the comparison of the data, the values of labile phosphate compounds in the two studies, extrapolated to the same size of brain sample, are identical.

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SUMMARY

The effect of chlorpromazine on brain levels of adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, and creatine phosphate was determined. The levels of the compounds in depressed animals killed by whole body immersion in liquid oxygen were identical to those of saline-injected controls. Killing by decapitation and subsequent immersion of the head in liquid oxygen resulted in a greater fall in levels of adenosine triphosphate and creatine phosphate in brains of controls than in the drug-treated group. With this procedure, creatine phosphate and adenosine triphosphate levels of chlorpromazine-depressed animals were significantly greater than, and adenosine monophosphate levels were significantly lower than, those of the controls. Enzymic studies on brains of animals depressed with chlorpromazine revealed that creatine phosphokinase activity and the activities of enzymes catalysing the dephosphorylation of adenine nucleotides were unaltered by the drug. The results suggest that chlorpromazine in some way depresses the utilization of brain adenosine triphosphate, so that a given stimulus results in less utilization of labile phosphate. The normal levels of labile phosphate compounds in brains of chlorpromazine-depressed animals indicate that the biochemical effects of chlorpromazine involving interference with oxidative enzyme systems and uncoupling of oxidative phosphorylation are unrelated to the primary pharmacological action of the drug.

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AMINO ACID AND PROTEIN METABOLISM—VI CEREBRAL COMPARTMENTS OF GLUTAMIC ACID METABOLISM

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IN RECENT studies of glutamic acid metabolism *in vivo*, the fate of tracer amounts of [^{14}C]-labelled glutamic acid administered intravenously to rats and mice was investigated in experiments of short duration (LAJTHA, BERL and WAELSCH, 1959). The major portion of the amino acid apparently entered brain, liver, kidney and muscle as the acid and conversion to the amide was not an essential prerequisite for exchange across the blood-brain barrier. The metabolic changes which then ensued were quite rapid, for within 2 min after injection of [^{14}C]glutamic acid, the glutamic acid, glutamine, and glutathione* fractions of brain, liver and kidney and the γ-aminobutyric acid fraction of brain were significantly labelled; the specific activity of the organ glutamine was closest to that of the dicarboxylic acid.

In many experiments with [^{14}C]glutamic acid the specific activity of plasma glutamine was higher than that of glutamine and glutamic acid in the brain and liver. Such a result would be obtained if the plasma glutamine were being formed in organs other than those analysed, or if the glutamine were synthesized from [^{14}C]glutamic acid of higher specific activity than that resulting from the mixing of the administered labelled amino acid with the organ glutamic acid. In addition, the newly formed amide may have been released to the blood before being equilibrated with the organ glutamine. The latter explanation suggests cellular compartmentalization of glutamine synthesis.

In this report data are presented on the metabolism of [^{14}C]glutamic acid after intracisternal administration of [^{14}C]glutamic and aspartic acids and [^{14}C]glutamine to rats and monkeys. As in the study of protein turnover and the previous investigations, the animals were exposed for only short periods of time to the isotopic amino acids in order to avoid equilibration of the label and to study the initial fate of the administered glutamic acid.

EXPERIMENTAL

Material. Uniformly labelled [^{14}C]glutamic acid (11 μc/μmole) and uniformly labelled [^{14}C]-aspartic acid (5 μc/μmole) were obtained from Nuclear Chicago Corp. Uniformly labelled [^{14}C]-glutamine (0·93 μc/μmole) was obtained from Merck and Co. of Canada. The purity of the labelled compounds was tested by column and paper chromatography.

Animal and organ preparations

Intracisternal injection of rat. Sprague-Dawley rats approximately 100 g in weight were used. Under light ether anaesthesia 0·02 ml of a neutralized amino acid solution was injected in the midline

* The abbreviations used are: GSH, glutathione; GABA, γ-aminobutyric acid; TCA, trichloroacetic acid.

at the external occipital protuberance, as described by JEFFERS and GRIFFITH (1949). Following recovery from anaesthesia the animals showed no ill effects from the intracisternal injection. Control injections with India ink indicated that the amino acid solution was placed into the cisterna magna. At the end of the experimental period the animals were decapitated. In the experiments of 15 sec and 1 min duration (Table 2), the whole head was dropped into liquid nitrogen, and the frozen brain was chiselled out of the skull. In experiments of longer duration the brain and liver were removed, rinsed in ice-cold saline, blotted dry with filter paper, and frozen in dry ice. Less than 1 min passed between the killing of the animal and the freezing of the brain. In other experiments the brain was sectioned into six parts on a petri dish resting on cracked ice. The cerebellum was removed, and the pons and medulla were dissected together. The cerebrum was cut transversely into two equal parts, each of which was cut again horizontally so that four cerebral parts were obtained, each containing tissue from both hemispheres. The six sections of brain were placed in dry ice and weighed in the frozen state. When blood plasma was analysed it was collected in heparinized tubes, centrifuged for 1 min at 18,000 **g**, and the plasma was frozen in dry ice.

Intracisternal injection of monkey. A Java monkey (*Macacca irus*) was maintained under ether anaesthesia. The skull was opened and the brain exposed. An 18-gauge needle was placed in the cisterna magna, 0·5 ml of spinal fluid was withdrawn, and 0·4 ml of a neutralized [^{14}C]glutamic acid solution in saline was injected. Ten minutes after the injection, the brain was removed and dissected as indicated in Table 4*. The dissected areas of tissue were frozen in dry ice.

In all experiments the frozen tissues were homogenized in cold 5% TCA and centrifuged. The procedure was repeated twice, and the extracts were combined. The ratio of tissue to total volume of extract was 1:10 in the case of the organs. Plasma was brought to a concentration of 5% TCA by the addition of 50% TCA, and the precipitate was re-extracted twice with 5% TCA to give a final ratio of plasma to extract of 1:3. The amounts of amino acid injected are given in the tables.

Separation of the amino acids. The methods of separation of the amino acids were as previously described (LAJTHA, BERL and WAELSCH, 1959; BERL and WAELSCH, 1958), with the exception that AG 1-x8 resin (purified Dowex 1, Bio-Rad Laboratories, Richmond, California) was used instead of Amberlite X E 59. The AG 1 resin (equal portions of 200–400 mesh and 400 mesh) was washed three times with 5 vols. of 2 N-NaOH, then three times with 5 vols. of 2 N-acetic acid, followed by 1 vol. of water. It was stored under an equal volume of 2 N-acetic acid. The column diameter was 10 mm; 25 ml of resin bed were used. To each column were added 10 ml of tissue extract. The amino acids were eluted from the column with the following successive portions of 0·5 N-acetic acid; 1 ml plus 14 ml (neutral amino acids and glutamine fraction); 8 ml (discarded); 17 ml (glutamic acid fraction). In this procedure aspartic acid remains on the column but can be eluted with acetic acid. The procedure was modified in the experiments in which [^{14}C]glutamine or [^{14}C]aspartic acid was injected into the cisterna magna of rats (Tables 5, 6, and 7). Glutamic acid, aspartic acid, and GSH were separated directly on columns of AG 1-x4 200–400 mesh. The resin was converted to the acetate form by suspending it for 20–30 min in 2 vols. of 2 N-acetic acid, separated on a Buchner funnel, and washed without suction with 5 vols. of 2 N-acetic acid. It was suspended in an equal volume of 2 N-acetic acid and allowed to stand overnight. Columns 20 × 0·7 cm were prepared, and 5 ml of 0·05 N-acetic acid were passed through each column. Two volumes of tissue extract were neutralized (pH 7 ± 0·1), and *p*-chloromercuriphenylsulphonic acid was added and diluted to 3 vols,. as previously described (BERL and WAELSCH, 1958). Three ml portions were passed through each column. The columns were treated consecutively with 8 ml (1·0 ml + 5·0 ml + 2·0 ml) of 0·05 N-acetic acid, 12 ml of 0·1 N-acetic acid, 10 ml of 0·3 N-acetic acid, and 12 ml of 0·2 M-sodium citrate buffer, pH 2·5. Glutamine and GABA were eluted with the first 6 ml of 0·05 N-acetic acid, glutamic acid in the 5–10 ml fraction of 0·1 N-acetic acid, the aspartic acid in the 2–8 ml fraction of 0·3 N-acetic acid, and the GSH in the 6–12 ml fraction of the sodium citrate buffer. Glutamine and GABA were separated on AG 50-x4 200–400 mesh (purified Dowex 50, Bio-Rad Laboratories, Richmond, California). The resin was converted to the sodium form by suspension in an equal volume of 2 N-NaOH, collected on a Buchner funnel, and washed consecutively without suction with 5 vols. of 2 N-sodium hydroxide, 5 vols. of water, and 5 vols. of 0·3 M-sodium citrate buffer pH 3·25. It was stored under an equal volume of buffer. Columns of resin measuring 12 × 0·7 cm were prepared and washed with an additional 5 ml of 0·3 M-sodium citrate buffer (pH 3·25). The pH of the 0·05 N-acetic acid eluates (6 ml each) from the AG 1 columns was reduced to 2·0–2·3 by the addition of 0·2 ml of N-HCl; 6 ml portions were

* We would like to thank Dr. L. ROIZIN for help in the experiment with the monkey.

passed through each AG 50 column, and the amino acids were washed into the columns with two 0·5 ml portions of 0·01 N-HCl. The columns were treated with 9 ml of 0·3 M-sodium citrate buffer (pH 3·25); glutamine was eluted in the 4–8 ml fraction. The buffer was changed to 0·35 M-sodium citrate, pH 5·30, and GABA was eluted in the 4–6 ml fraction. The fractions containing glutamic acid, aspartic acid, GSH, and GABA gave single spots when analysed by paper chromatography. Therefore, their concentrations were determined by the ninhydrin method (MOORE and STEIN, 1954*a*). The glutamine fraction was not pure and the amino acid was isolated as the glutamic acid from AG 1 columns after prior hydrolysis in 2 N-HCl for 2 hr at 100°. N-acetyl aspartic acid was eluted with the GSH fraction. Hydrolysis for 2 hr at 100° in 2 N-HCl released the glutamic acid from the GSH and the aspartic acid from the N-acetyl aspartic acid. Asparagine was eluted with the glutamine. It was isolated as aspartic acid after hydrolysis in 2 N-HCl at 100° for 2 hr. The hydrolysates were evaporated to dryness in a flash evaporator at 50° *in vacuo*, dissolved in a small volume of water, and desalted on AG 50 as described by WINZLER, MOLDAVE, RAFELSON and PEARSON (1952). The resultant ammonia eluate was concentrated to dryness and the residues were dissolved in 2·2 ml of water added to the evaporation flask. Two ml of the neutralized solution was chromatographed on AG 1 columns as described above. Glutamic acid was eluted in the 7–12 ml fraction of 0·1 N-acetic acid (14 ml were used) and aspartic acid in the 5–10 ml fraction of 0·3 N-acetic acid. The sodium citrate buffers (Table 1) are modifications of those described before by MOORE and STEIN (1954*b*) and MOORE, SPACKMAN, and STEIN (1958). The radioactivity in the samples was determined as previously described (LAJTHA, BERL and WAELSCH, 1959).

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TABLE 1.—PREPARATION OF SODIUM CITRATE BUFFERS

Citric acid H_2O (g)	NaOH 97% (g)	HCl 36% (ml)	Final sodium concentration (molar)	Final pH
21	8·3	14·7	0·20	2·50 ± 0·02
24·2	12·4	18·5	0·30	3·25 ± 0·02
24·55	14·4	6·7	0·35	5·30 ± 0·02

Final volume = 1l.

RESULTS

Intracisternal administration of glutamic acid. Cerebral glutamine synthesis was investigated in rats killed at short intervals after injection of [^{14}C]glutamic acid into the cisterna magna; blood plasma, brain and liver were analysed (Table 2). The specific activity of brain glutamine was consistently higher than that of plasma glutamine. Therefore, the glutamine in the brain could not have originated from the blood but must have been synthesized in the brain. The rate of conversion of glutamic acid to glutamine is very rapid. After 15 sec (the shortest time required for injection of the amino acid and freezing of the head by dropping it into liquid N_2) the activity of the glutamine is almost half that of the glutamic acid, in 1 min the glutamine specific activity is already $1\frac{1}{2}$ times that of the glutamic acid, and in 5 min the ratio is about 5 to 1. During the first 5 min both the total count and specific activity of the glutamic acid decrease while the radioactivity of the glutamine increases; but the combined radioactivity per gram of tissue contained in glutamic acid and glutamine remains constant. After 5 min the specific activity of the glutamine decreases faster than that of the glutamic acid with a resultant net loss in radioactivity; the total radioactivity decreases to about two-thirds within 30 min and to about one-half within 1 hr.

To determine whether or not the rapid glutamine formation occurs throughout the brain, the organ was sectioned following intracisternal injection of [^{14}C]glutamic acid, and the radioactivity of glutamic acid, glutamine, GSH, and GABA in each part was determined (Table 3). The specific activities of the metabolites were greatest in the sections of brain nearest to the site of injection, e.g. cerebellum, pons-medulla.

TABLE 2.—CEREBRAL GLUTAMIC ACID METABOLISM AFTER INTRACISTERNAL ADMINISTRATION OF [^{14}C]GLUTAMIC ACID

Time (min)	Substrates	Specific activity (counts/μmole/min)			Ratio Glu-NH_2/Glu	Total counts in 1 g fresh tissue	
		Plasma	Brain	Liver	Brain	Brain	Brain Total / (Glu + Glu-NH_2)
1/4	Glu*		7700		0·43	77,000	92,000
	Glu-NH_2*		3300			15,000	
1	Glu	180,000	5500	200	1·3	51,000	84,000
	Glu-NH_2	1200	7300	66		33,000	
2	Glu	130,000	3200	800	3·8	32,000	91,000
	Glu-NH_2	2200	12,000	600		59,000	
5	Glu	34,000	2700	700	5·2	27,000	97,000
	Glu-NH_2	6100	14,000	760		70,000	
15	Glu	8900	2200	190	5·0	22,000	77,000
	Glu-NH_2	6500	11,000	280		55,000	
30	Glu	3300	2100	180	3·5	21,000	58,000
	Glu-NH_2	870	7300	240		37,000	
60	Glu	1500	1900	61	2·6	19,000	44,000
	Glu-NH_2	140	5000	130		25,000	

* Glu = Glutamic Acid
Glu-NH_2 = Glutamine
In each experiment two rats were injected via the cisterna magna with uniformly labelled [^{14}C]glutamic acid (0·02 ml of a solution containing 40 μc and 4 μmole per ml in 0·9% saline). Each figure is the average of two experiments.

Although the activities of the four compounds varied considerably from one part of the brain to another, in each section the specific activity of glutamine was three to five times as great as that of glutamic acid; the specific activity of the GSH was about equal to that of the glutamic acid. The activity of GABA was one-tenth to one-third that of glutamic acid. This experiment was repeated with a monkey in order to obtain various areas large enough for analysis (Table 4). The observations were similar to

Table 3.—Cerebral metabolism of glutamic acid after intracisternal administration of [^{14}C]glutamic acid

Brain area	Counts/min/μmole				Relative specific activity (glutamic acid = 1·0)		
	Glutamic acid	Glutamine	Glutathione	γ-Amino butyric acid	Glutamine	Glutathione	γ-Amino butyric acid
Cerebellum	7000	37,000	7200	2400	5·3	1·0	0·34
Pons-medulla	14,000	70,000	16,000	3200	5·0	1·1	0·23
Posterior superior cerebrum	1600	5100	1600	460	3·2	1·0	0·29
Anterior superior cerebrum	260	1400	380	80	5·4	1·5	0·31
Posterior inferior cerebrum	4600	18,000	4300	560	3·9	0·93	0·12
Anterior inferior cerebrum	1800	8700	1500	230	4·8	0·83	0·13

For experimental details see Table 2. Rats were killed after 2 min. Average of four experiments.

those obtained in the rat. There was considerable variation from area to area, with the highest activities in the area nearest the site of injection, e.g. upper spinal cord, pons-medulla, and cerebellum. Except in blood and cortical white matter, the specific

Table 4.—Cerebral glutamic acid metabolism in the monkey after intracisternal administration of [^{14}C]glutamic acid

	Counts/min/μmole			
	Glutamic acid	Glutamine	Glutathione	γ-Amino-butyric acid
Frontal Cortex	430	1600	370	51
Parietal Cortex	82	160	77	24
Temporal Cortex	540	5300	780	310
Occipital Cortex	76	170	82	(9)
Lenticular nucleus	440	1000	490	46
Thalamus	210	370	190	22
Hypothalamus + Optic chiasm	4300	6300	3300	340
Caudate nucleus	56	100	48	(1)
Cerebellum	5600	21000	3700	3000
Mesencephalon	2400	12000	2800	440
Pons	14000	78000	11000	4200
Medulla	26000	110000	15000	6400
Frontal Lobe White Matter	220	170	120	(16)
Corpus Callosum	220	480	200	64
Rest of Brain	380	510	280	110
Spinal Cord, Upper Part	66000	310000	32000	18000
Spinal Cord, Lower Part	25000	52000	10000	1400
Blood	10000	310	270	—

Java monkey (*Macacca irus*) was injected via the cisterna magna with 0·4 ml of [^{14}C]glutamic acid (50 μc and 5 μmole/ml in physiological saline). Under light ether anaesthesia the skull was opened and 10 min after injection the brain was removed and dissected (total time after injection 20 min). Spinal cord removed 35 min after injection.

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TABLE 5.—METABOLISM OF GLUTAMINE AFTER INTRACISTERNAL ADMINISTRATION OF [^{14}C]GLUTAMINE

Brain area	Counts/min/μmole					Relative specific activity (glutamic acid = 1·0)			
	Glutamic acid	Glutamine	GSH	GABA	Aspartic acid	Glutamine	GSH	GABA	Aspartic acid
Cerebellum	860	9900	1500	780	620	12	1·7	0·9	0·7
Pons-medulla	930	12000	2000	540	490	13	2·2	0·6	0·5
Posterior superior cerebrum	330	3900	440	160	220	12	1·3	0·5	0·7
Anterior superior cerebrum	300	4700	530	270	170	16	1·8	0·9	0·6
Posterior inferior cerebrum	140	2400	280	110	110	17	2·0	0·8	0·8
Anterior inferior cerebrum	900	8900	1100	540	450	9·9	1·2	0·6	0·5

Rats were injected with 0·02 ml of [^{14}C]glutamine (50 μc and 12 μmole per ml). The animals were killed after 2 min. Tissue from two animals was pooled for each experiment. The above is the average of three experiments.

activity of glutamine was approximately two to five times as high as that of glutamic acid. In many areas the specific activity of GSH approached or actually surpassed that of glutamic acid. As in the rats, the specific activity of GABA was always less than that of glutamic acid. Similar results were obtained after subarachnoid administration of [^{14}C]glutamic acid to rats.

Intracisternal administration of glutamine. Cerebral metabolism of glutamine was examined by administering [^{14}C]glutamine into the cisterna magna of rats. The administered glutamine entered the metabolic reactions rapidly and the compounds analysed—glutamine, glutamic acid, GSH, GABA and aspartic acid—all contained significant radioactivity (Table 5). The specific activity of glutamine remained considerably higher than that of glutamic acid. In these experiments the ratios of specific

TABLE 6.—METABOLISM OF ASPARTIC ACID IN RAT BRAIN AFTER INTRACISTERNAL ADMINISTRATION OF [^{14}C]ASPARTIC ACID

	counts/min/μmole		
	Brain (2 min)	Brain (5 min)	Plasma (5 min)
Glutamic acid	1600	2700	1800
Glutamine	8000	13000	420
Aspartic acid	55000	34000	35000
Asparagine	960	2400	91
Glutathione	570	1600	
γ-Aminobutyric acid	180	1000	
N-Acetyl aspartic acid	38	24	

Young male Sprague Dawley rats (90–110 g) were injected via the cisterna magna with 0·02 ml of an [^{14}C]aspartic acid solution containing 50 μc and 10 μmole/ml.

The above is the average of 2 experiments; 2 animals were used in each experiment.

activities of GSH and GABA to that of glutamic acid were greater than in those in which glutamic acid was administered, and actually the radioactivity of GSH was significantly above that of glutamic acid. The activity of the aspartic acid was close to that of GABA (specific activity ratios to glutamic acid 0·5–0·9).

Intracisternal administration of aspartic acid. After the intracisternal injection of [^{14}C]aspartic acid the label appeared rapidly in glutamic acid and all its metabolic derivatives (Tables 6 and 7). When the specific activities of glutamine, GABA and GSH are each related to that of glutamic acid the picture is similar to that after the administration of glutamic acid, with a specific activity of the amide up to 5 times that of glutamic acid, and the specific activity of GSH intermediate between that of GABA and the dicarboxylic amino acid. The specific activity of asparagine is up to 10 per cent of that of aspartic acid. The low specific activity of acetyl aspartic acid should be noted (JACOBSON, 1958; GOLDSTEIN, 1959). The specific activities of aspartic and glutamic acids in the plasma attest to the rapid movement of the amino acids into the blood.

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TABLE 7.—CEREBRAL METABOLISM OF ASPARTIC ACID AFTER INTRACISTERNAL ADMINISTRATION OF [^{14}C]ASPARTIC ACID

Brain area	Specific activity (counts/min/μmole)						
	Aspartic acid	Glutamic acid	Asparagine	N-Acetyl aspartic acid	Glutamine	γ-Aminobutyric acid	Glutathione
Cerebellum	160000	3400	3600	99	16000	680	1800
Pons-medulla	70000	4500	890	64	18000	330	880
Posterior superior Cerebrum	58000	1700	1000	34	9400	260	700
Anterior superior Cerebrum	4900	170	470	10	610	18	74
Posterior inferior Cerebrum	60000	1900	750	50	7700	120	600
Anterior inferior Cerebrum	25000	680	360	21	3300	61	280
Whole Brain	55000	1600	960	38	8000	180	570

0·02 ml of an aspartic acid solution (50 μc and 10 μmole in 1 ml 0·9% saline) were injected in the cisterna magna of young (100 g) rats. The animals were killed after 2 min.

DISCUSSION

Compartmentation in glutamine formation

After the intravenous administration of [^{14}C]glutamic acid, plasma glutamine showed a higher specific activity than the glutamic acid and glutamine of brain and liver in a number of experiments. This finding was interpreted as indicating a compartment of glutamine synthesis with preferential use of the administered amino acid. Similarly, after intracisternal administration of [^{14}C]glutamic acid, the glutamine of the brain of rats and monkeys showed a considerably higher specific activity than its precursor, the dicarboxylic acid.

Effect of metabolism on the specific activity of glutamic acid

Postulation of the compartmentalization of a metabolic event such as glutamine synthesis based on inverse labelling of precursor and product, as in the experiments of the type undertaken, is justified only after the possibility has been excluded that the results can be explained by different rates of the pertinent metabolic reactions. Of particular importance are the reactions which would, at a rapid rate, dilute the labelled glutamic acid. The major reactions of interest in this respect are: (a) the rates of metabolism of glutamic acid and glutamine; (b) the rate of turnover of proteins which would supply unlabelled glutamic acid and glutamine for dilution of the labelled compound; (c) contribution from carbohydrate metabolism.

(a) *Rates of metabolism of glutamic acid and glutamine.* In the experiments of 1–60 min duration (Table 2), cerebral glutamine showed a higher specific activity than brain glutamic acid. This is true for all parts of the brain after intracisternal administration in both rats and monkey, with the exception of the frontal lobe white matter in the monkey. That this finding is not due to a dilution or removal of the labelled glutamic acid during the first 5 min of the experiment is apparent from the constancy of the sum of radioactivities of glutamic acid and its amide per gram of brain. During this time period the radioactivity is redistributed between the acid and the amide. From the 5th to the 60th minute the decrease in specific activity of glutamine is 65 per cent as compared with 30 per cent for the glutamic acid, a finding which indicates that the drop in total radioactivity per gram of tissue is to a larger extent due to the metabolism or loss of glutamine.

(b) *Contribution from protein turnover.* The finding of the constancy of the sum of radioactivities in glutamine and glutamic acid during the first 5 min of the experiment is supported by calculations of the possible maximal contribution to the glutamic acid pool by glutamic acid derived from protein metabolism.

By assuming an average half-life time of 3 days of the total brain proteins, as determined in short-time experiments (LAJTHA, FURST, GERSTEIN and WAELSCH, 1957; LAJTHA, 1959) for the metabolically most active protein fraction, the contribution of unlabelled glutamic acid or glutamine to the pool of free amino acids may be calculated maximally as 10 mg for each compound per hr per 100 g fresh brain (12 g protein per 100 g; 10% glutamic acid and glutamine respectively). Such figures would amount to a contribution during 5 min of 0·5 or 1·5%, respectively, of free unlabelled glutamic acid or glutamine from protein metabolism and this could not be responsible for the dilution of radioactivity observed. These values are considered to be maximal since only a fraction of the brain protein will have a half-life time as low as 3 days.

(c) *Contribution from carbohydrate metabolism.* In order to calculate the maximal possible dilution of glutamic acid from unlabelled ketoglutarate derived through the citric acid cycle, the unlikely assumption may be made that all ketoglutarate is converted into glutamic acid. With a utilization of 150 μmole of glucose per 100 g of brain tissue per 5 min, about 150 μmole of unlabelled ketoglutaric acid would be added to the 1000 μmole of glutamic acid already present.

Therefore, neither metabolic dilutions of the glutamic acid, nor the contributions of glutamic acid from protein metabolism and the citric acid cycle, alone or combined, could explain a 5-fold dilution of glutamic acid during the first 5 min of the experiment.

It may, therefore, be concluded that the higher specific activity of brain glutamine relative to glutamic acid after intracerebral administration is the result of the conversion of a small amount of glutamic acid of high specific activity to glutamine before dilution by the total amount present in the tissue.

Compartmentation in the formation of GSH and of GABA.

Two minutes after intracisternal injection of [^{14}C]glutamic acid, the specific activity of GSH approximates, and in some instances, exceeds the specific activity of glutamic acid. If GSH draws its glutamic acid from a pool of higher than average activity, its specific activity may come close to that of glutamic acid or exceed it. In no instance does the specific activity of GABA exceed that of glutamic acid. This finding suggests that GABA is derived either from the total pool of the amino acid or from a very small amount of glutamic acid with higher than average activity, so that the dilution with unlabelled tissue GABA would lower the specific activity of the newly formed compound below that of glutamic acid.

Metabolism of administered glutamine

Of particular interest are the results after intracisternal administration of [^{14}C]-labelled glutamine. In these 2 min experiments the ratio of radioactivity of the glutamic acid moiety of glutathione to cerebral glutamic acid is higher than in any experiments in which labelled glutamic acid itself was administered, and that of GABA approaches the specific activity of the dicarboxylic acid. While these results, in view of the evidence on the mechanism of GSH synthesis obtained with the aid of cell free systems (JOHNSTON and BLOCK, 1951; FODOR, MILLER, NEIDLE and WAELSCH, 1953), should not be taken to indicate that glutamine as such takes part directly in the synthesis of the tripeptide, they seem to support the previous finding that glutamine penetrates with greater ease into the tissue than glutamic acid (SCHWERIN, BESSMAN and WAELSCH, 1950). It might migrate at a faster rate to the compartment of GSH synthesis or glutamic acid decarboxylation. These results support the contention that both GSH and GABA formation are intracellular processes.

Localization of the compartments of glutamic acid metabolism

Since brain glutamine is of higher specific activity than blood glutamine the former must have originated in the brain and not entered from the blood (Tables 2 and 5). On the other hand, after intracisternal injection the specific activity of the plasma glutamic acid was always higher than that of brain or liver glutamic acid or amide; this attests to the rapid egress of the acid from the site of injection.

The finding of high specific activity of cerebral glutamine after intracisternal administration of glutamic acid could be interpreted as demonstrating conversion of the amino acid to the amide at the site of administration, but the similarity of results obtained in 17 parts of the brain after intracisternal administration of [^{14}C]glutamic acid to a monkey argues against such a possibility.

In view of the easy penetration of glutamine into brain tissue (Table 4), it might be supposed that the compartment of amidation of administered [^{14}C]glutamic acid is located in the cerebral surface membranes or the ependymal lining. All further conversions, such as to GSH or GABA, would then be the result of the metabolism of the labelled amide. In this connection it is of interest that the ratios of specific

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activities of glutamine to GSH or GABA are of the same order of magnitude whether [^{14}C]glutamic acid or [^{14}C]glutamine is administered intracisternally. Although not decisive, the following evidence argues against this possibility. It was shown some years ago that glutamic acid and its amide are absorbed through the gut as such (BESSMAN, MAGNES, SCHWERIN and WAELSCH, 1948) and the same has been demonstrated recently for the entrance of the two compounds from the circulating blood into the brain (LAJTHA, BERL and WAELSCH, 1959). These findings cannot exclude completely the possibility of a limited interconversion with succeeding metabolism of glutamine.

The strongest evidence for conversion of glutamic acid to glutamine within the brain tissue may be derived from the experiments in which [^{14}C]aspartic acid was administered intracisternally. Within 5 min glutamic acid and all its metabolic derivatives were labelled in ratios similar to those found after the administration of [^{14}C]glutamic acid. It would have to be assumed that formation of oxalacetate from aspartic acid by transamination, conversion of oxalacetate to ketoglutarate, and amination of the latter to glutamic acid with succeeding amidation to glutamine proceed in the arachnoid, pial or ependymal cells, and not in the brain tissue proper. Recent experiments in which the fate of [^{15}N]ammonia after carotid infusion was studied demonstrated the presence of a compartment for glutamine synthesis from intracellularly synthesized glutamic acid (TAKAGAKI, BERL, CLARKE, PURPURA and WAELSCH; 1961). When, after a short time interval following intracisternal administration of [^{14}C]leucine and [^{14}C]lysine to rats, the brain is sectioned into slices, label is found in all slices irrespective of the extent of contact with the outer or inner surfaces of the organ (LAJTHA and MELA, 1961). While not decisive, all the evidence points to the conclusion that the compartment of glutamine synthesis from intracisternally administered glutamic acid is located within the cells of the brain tissue.

Size of the compartment

The data of the experiments recorded in Table 2 may be utilized for an approximate estimate of the size of the compartment of glutamic acid utilized for glutamine synthesis. The ratios of specific activities of glutamic acid to glutamine of 1 : 5 suggest that as a maximal value 20 per cent of the glutamic acid is converted to glutamine. The true size of the active glutamic acid pool will be a fraction of this value and depend on the size of the active glutamine pool. If only for the purpose of this estimation the unlikely assumption is made that the active pool of glutamine corresponds to the total tissue glutamine with an approximate half-life time of 30 min (calculated from Table 2, 5–30 min interval), the active glutamic acid pool would be roughly 2 per cent of the tissue glutamic acid. Any smaller active glutamine pool would decrease correspondingly the size of the pool of glutamic acid being converted to glutamine. The conversion of such a fraction of glutamic acid to glutamine, if by an energy-requiring process and not an exchange reaction, would represent only a minor drain on the adenosine triphosphate supply of the tissue.

Compartmentation and calculations of turnover rates

Heterogeneous distribution of metabolites and compartmentalization of metabolic events has been postulated in recent years for various aspects of intermediary

metabolism. There is an increasing amount of data suggesting metabolic compartments in amino acid metabolism. The possibility of separate pools of amino acid for synthetic and storage purposes has been pointed out (COWIE and WALTON, 1956). 'Active' and 'storage' pools of glutamine were found in the metabolism of carrot root extracts (STEWARD, BIDWELL and YEMM, 1956), and similarly, proline seems to be present in a heterogeneous intracellular pool as far as its incorporation into proteins is concerned (GREEN and LOWTHER, 1959).

The demonstration of the presence of compartments in the metabolism of glutamic acid and its derivatives is of considerable consequence for the estimation and significance of the rates of turnover for these metabolites. It becomes obvious that with the presence of metabolic compartments with a rapid metabolism of small amounts of a metabolite in the presence of large concentrations of the same compound, over-all turnover rates lose their meaning since they are a composite of the turnover rates in various metabolic compartments. The classical precursor-product relationship used for estimation of turnover rates (ZILVERSMIT, ENTENMAN and FISHLER, 1943) will hold only in long-term experiments in which the labelled compound will have penetrated all compartments. In those cases in which in short-time experiments the product has a higher specific activity than the precursor, a compartmentalization of the metabolic event may be suspected, while in all those cases where under the same conditions the product has a lower specific activity than the precursor it is at present impossible to decide whether we are dealing with the conversion of a small amount of precursor or a slow rate of conversion. It may be concluded that the turnover rates in compartments of active metabolism surpass previous estimates. Definite statements as to the rates of metabolism in the various compartments *in vivo* will be possible only after the size of the various compartments can be estimated. Since the compartments are not closed to each other, the migration of substances from one compartment to another further complicates the picture. Only after a clear understanding of the significance of metabolic compartments will there emerge an approximate picture of *in vivo* metabolism and its controlling factors (see WAELSCH, 1960).

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SUMMARY

In order to study the compartments of glutamic acid metabolism in brain, [^{14}C]-glutamic acid, [^{14}C]aspartic acid, and [^{14}C]glutamine were administered intracisternally to animals. Following intracisternal administration of [^{14}C]glutamic acid, the glutamine of the brain of rats and monkeys showed, in short-time experiments, a considerably higher specific activity than its precursor, glutamic acid—the specific activity of GSH being close to, and that of GABA, lower than that of glutamic acid.

Following the injection of [^{14}C]glutamine into the cisterna magna of rats, the specific activity of the glutamic acid in GSH of the brain was greater than, while that of GABA was close to, that of glutamic acid. After the intracisternal administration of [^{14}C]aspartic acid cerebral glutamic acid and its metabolic derivatives became labelled rapidly in ratios similar to those found after the administration of [^{14}C]-glutamic acid. The low specific activity of N-acetyl aspartic acid attests to the metabolic inertness of this compound.

The findings support the conclusion that after intracisternal administration of glutamic acid, the labelled glutamine is derived in the brain from a small glutamic

acid pool of higher than average specific activity. This active pool is estimated tentatively as corresponding to less than 20 per cent and probably in the neighbourhood of 2 per cent of the total tissue glutamic acid. Similar conclusions can be drawn for the formation of GSH in the tissue.

The significance of this type of experiment for the study of *in vivo* compartmentalization of metabolic events and for an estimation of true metabolic rates is discussed.

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METABOLIC AND PHARMACOLOGICAL EFFECTS OF INJECTED AMINO ACIDS AND AMMONIA ON CORTICAL EPILEPTOGENIC LESIONS*

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RAPID freezing and rewarming of a small area of neocortex in the locally anæsthetized succinyl choline-paralysed cat initiates a sequence of complex alterations that result in (a) localized destruction of the blood-brain barrier (PURPURA, GIRADO, SMITH and GOMEZ, 1958*a*, *b*); (b) the development of focal paroxysmal discharges (MORRELL and FLORENZ, 1958; SMITH and PURPURA, 1960); and (c) changes in amino acid metabolism (BERL, PURPURA, GIRADO and WAELSCH, 1959). In the last-mentioned study, it was found that focal seizure activity in lesion sites was accompanied by decreases in glutamic acid, glutamine, and GSH§ that were significantly greater than those occurring in non-epileptogenic lesions. In striking contrast to the decreases observed in these metabolites, no significant differences in GABA content were found in epileptogenic and non-epileptogenic lesions and intact cortex.

The present study is a further inquiry into the relationship between brain amino acid metabolism and electrocortical events. Advantage is taken of the absence of the blood-brain barrier in freezing lesions of neocortex to analyse the effects of intravenously administered amino acids on the changes in amino acid metabolism encountered in such lesions, and to define, if possible, the relationship between the immediate pharmacological actions of systemically administered GABA and glutamic acid and their metabolic activities. Data are also presented on the physiological and metabolic effects of injected ammonia (ammonium acetate) that permit some answer to the question raised by BERL *et al.* (1959) concerning the role of NH_3 in the development of focal discharges. These data taken together with those on the effects of injected GABA and glutamic acid indicate that, whereas these amino acids tend to suppress low frequency discharges and increase the levels of glutamic acid, glutamine, and GSH to normal values, ammonium acetate infusions are singularly ineffective in altering the excitability of such lesions despite attainment of tenfold increases in tissue concentrations of NH_3.

MATERIAL AND METHODS

Locally anæsthetized, succinylcholine paralysed cats prepared in a manner similar to that described previously (PURPURA and GRUNDFEST, 1956, 1957) were used. Focal epileptogenic lesions, produced by brief application of an ethyl chloride spray to the exposed cortex, were allowed to develop regular

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§ Abbreviations used are: GSH, glutathione; GABA, γ-aminobutyric acid; EEG, electroencephalogram; ECG, electrocardiogram.

paroxysmal discharge patterns for 30 min to 3 hr before injection of GABA, glutamic acid, or ammonia. The metabolites were administered intravenously via an indwelling femoral vein canula. At the conclusion of the observation period, the region of cortex (posterior sigmoid gyrus) involved in the paroxysmal activity and the same area in the contralateral hemisphere were frozen *in situ* with solid CO_2 at the time when the animal was injected with a lethal dose of sodium pentobarbital.

In studies involving injections of glutamic acid and GABA, the methods of BERL and WAELSCH (1958) were utilized for determining tissue concentrations of glutamic acid, glutamine, GABA, and GSH. In the ammonium acetate injection studies, glutamic acid, aspartic acid, and GSH were separated on Ag 1*, and glutamine and GABA were separated on Ag 50* (*cf.* modified procedure, BERL, LAJTHA and WAELSCH, 1961). After GABA separation, tissue ammonia was eluted from the Ag 50 column with 0·38 M-sodium phosphate buffer (pH 6·5). The buffer had been freed from contaminating NH_3 by passing it through Ag 50 Na form (30–100 ml fraction from a 2 × 25 cm column). The tissue ammonia was present in the 2–6 ml fraction. Glutamic acid, aspartic acid, GSH, and GABA were determined by the ninhydrin method of MOORE and STEIN (1954). Ammonia was also determined by the ninhydrin method after absorption into 0·01 N-HCl in Cavett† diffusion flasks or Conway units. The glutamine was determined by the ferric hydroxamate method (BERL and WAELSCH, 1958).

RESULTS

Low-frequency, paroxysmal discharges localized to the lesion site were rapidly suppressed by systemically administered GABA; this finding confirms previous results (PURPURA, GIRADO, SMITH and GOMEZ, 1958*a*, *b*). With relatively large single injections of the amino acid (125 mg/kg) focal discharges were suppressed for experimental periods as long as 30 min (Fig. 1A). In other experiments, in which complex activity developed, suppression was either transient (1–5 min) or not observed at all (Fig. 1B). The pharmacological action of GABA thus depended on the rate and concentration of the injected amino acid and, most importantly, on the characteristics of the discharging lesion.

The average concentrations of glutamic acid, glutamine, and GSH in the lesion sites were higher after injection of GABA (Tables 1a and 1b) than those found previously in preparations exhibiting focal discharges for comparable periods of time (BERL *et al.*, 1959). It should be noted that contralateral areas with preserved blood-brain barrier had normal GABA levels although GABA levels increased as much as 250–450 per cent in the lesion sites.

The changes, after GABA administration, in concentration of glutamic acid and its metabolic derivatives and the effect on the paroxysmal activity of the lesions do not show a correlation in time. In Expts. 1 and 2 (Fig. 1A and Table 1a) no significant rise in GABA concentration was noticed in the lesion samples removed 5 and 15 min, respectively, after the administration of GABA despite the fact that the abnormal activity had been suppressed within 15 sec. Concentrations of glutamic acid, glutamine, and GSH in lesions removed 5 min after the administration of GABA were similar to those found in preparations which had received no GABA. On the other hand in lesions removed 15 min after GABA injection the concentrations of glutamic acid, glutamine, and GSH tended to rise to normal values. As shown by these experiments, 'simple' focal discharges are suppressed by GABA administration before there is an apparent rise in GABA tissue levels. When discharges are of a complex nature the tissue level may be raised 450 per cent without suppressing the abnormal activity (Fig. 1B; Table 1a, Expt. 6).

* Ag 1 and Ag 50 are purified Dowex 1 and Dowex 50 obtained from Bio-Rad Laboratories, Richmond, California.

† Obtained from Microchemical Specialities Co., Berkeley 3, California.

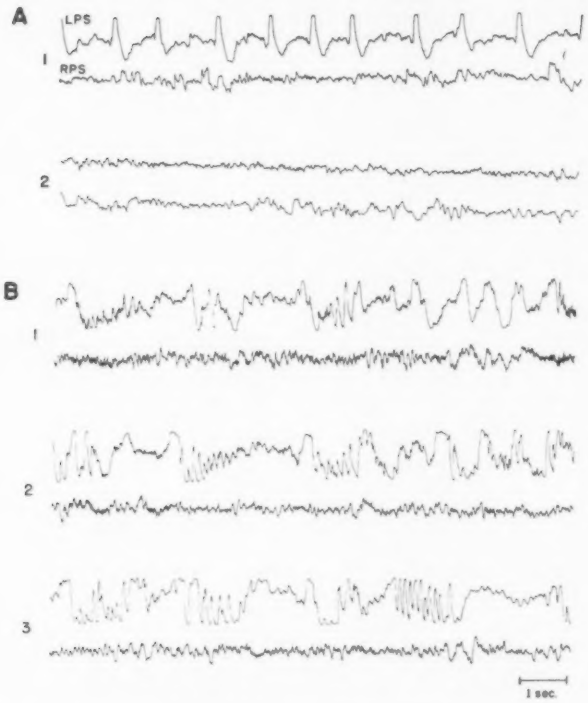

FIG. 1.—A1; *Upper channel:* Low-frequency focal paroxysmal discharges from left posterior sigmoid gyrus (LPS) freezing lesion.
Lower channel: Spontaneous electrocortical activity from right posterior sigmoid gyrus (RPS).
A2; 30 min after 125 mg/kg GABA. Focal discharges suppressed 15 sec after injection.
B1; *Upper channel:* LPS focal epileptogenic lesion. Activity of complex variety.
Lower channel: Control, RPS.
B2; 15 sec after 100 mg/kg, GABA, i.v.
B3; 5 min after additional 100 mg/kg GABA, i.v. GABA fails to suppress this type of focal epileptogenic activity.

Whereas systemically administered GABA rapidly suppressed low-frequency focal discharges of relatively 'simple' configuration,* glutamic acid similarly administered was considerably less effective in suppressing such activity. Not uncommonly, an initial injection of glutamic acid produced transient suppression of 'complex' discharges (Fig. 2) followed by return of paroxysmal activity sometimes of greater intensity than that observed before the injection. A second or third injection of glutamic acid might then completely suppress such discharges (Fig. 2).

As in the GABA injection experiments, following glutamic acid injection the average concentration of glutamic acid, glutamine and GSH approached values obtained in uninjured control areas (Tables 2a and 2b). In one animal the GABA

* The terms 'simple' and 'complex' are employed here as a matter of convenience and are not intended to convey the impression that pathophysiological processes underlying the low-frequency triphasic 'spike' (Fig. 1B) recorded in focal lesions are fundamentally dissimilar. Indeed, available data suggest that the 'simple' focal discharge represents the summated synaptic activities in a variety of complexly organized elements (SMITH and PURPURA, 1960).

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TABLE 1a.—CHANGES IN GLUTAMIC ACID, GLUTAMINE, GABA, AND GLUTATHIONE IN ETHYL CHLORIDE LESIONS AFTER I.V. ADMINISTRATION OF GABA

	Glutamic acid		Glutamine		GABA		GSH		GABA injected (mg/kg)	Effect of GABA on focal discharges	Period between last injection and tissue removal (min)
	μmoles/g										
Expt.	C*	L*	C	L	C	L	C	L			
1	8·2	4·0	4·9	2·7	1·6	1·7	1·9	1·0	80	Suppressed within 15 sec (Fig. 1A)	5
2	9·9	9·0	5·5	5·2	1·7	1·9	2·4	2·2	80	Suppressed within 15 sec	15
3	7·1	5·7	4·1	2·7	1·1	3·2	1·9	1·2	75; 17 min later 75	Suppressed within 15 sec then a return of activity. Suppressed partially after second injection	25
4	11·5	8·4	5·8	2·7	1·5	4·0	2·3	1·2	80; 15 min later 40	Suppressed within 15 sec	25
5	8·5	6·4	5·1	4·9	1·6	3·8	1·6	1·3	125	Suppressed within 15 sec	30
6	12·0	7·7	6·4	4·0	2·0	9·1	2·3	1·1	100; 4 min later 100	Not affected; spiking unusual in character (Fig. 1B)	30

* C = Control, L = Lesion

level in the lesion site was significantly higher than that in the control area. In preparations in which repeated injections of glutamic acid produced significant suppression of focal discharges, glutamic acid, glutamine, and GSH were increased to the ranges previously found in non-epileptogenic lesions (BERL *et al.*, 1959). Increases in glutamic acid levels above the normal range were never observed in lesion sites even after multiple glutamic acid injections in contrast to the increases in GABA after systemic GABA administration.

Intravenously administered glutamine had no significant effect on the discharge characteristics of focal lesions. Analyses of lesions removed from an animal after the

TABLE 1b.—CHANGES IN GLUTAMIC ACID, GLUTAMINE, GABA, AND GLUTATHIONE IN ETHYL CHLORIDE LESIONS AFTER I.V. ADMINISTRATION OF GABA
(% of control values-areas contralateral to lesion)

Expt.	Glutamic acid (54)*	Glutamine (51)*	GABA (95)*	GSH (47)*
1	49	55	106	53
2	100	95	112	92
3	80	66	291	63
4	73	47	267	52
5	75	96	238	81
6	64	62	455	48

* % of control values in lesions before administration of GABA (BERL, PURPURA, GIRADO and WAELSCH, 1959).

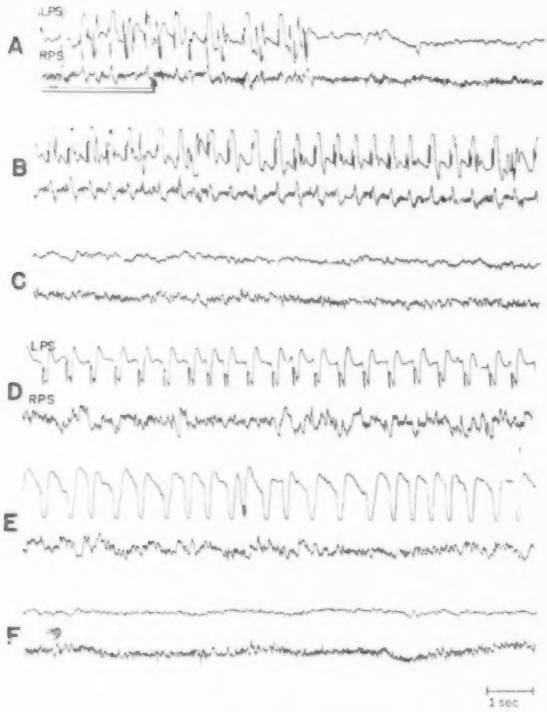

FIG. 2.—Effects of systemic glutamic acid on focal paroxysmal discharges. A—C and D—F, two different experiments. Upper and lower channels as indicated in Fig. 1.

A. Arrow denotes end of initial glutamic acid injection (100 mg/kg). Immediate suppression of complex focal discharges.
B. Few minutes after A. Focal discharges of greater intensity exhibiting transcallosal propagation.
C. 30 min after second injection of glutamic acid (100 mg/kg), which effectively eliminated focal discharges 1 min after injection.
D. Characteristics of focal discharges (LPS) prior to initial injection of glutamic acid (80 mg/kg).
E. Change in electrographic characteristics after the second injection (total 160 mg/kg).
F. Elimination of focal discharges after 5 successive injections of glutamic acid at 5 min intervals (total 400 mg/kg).

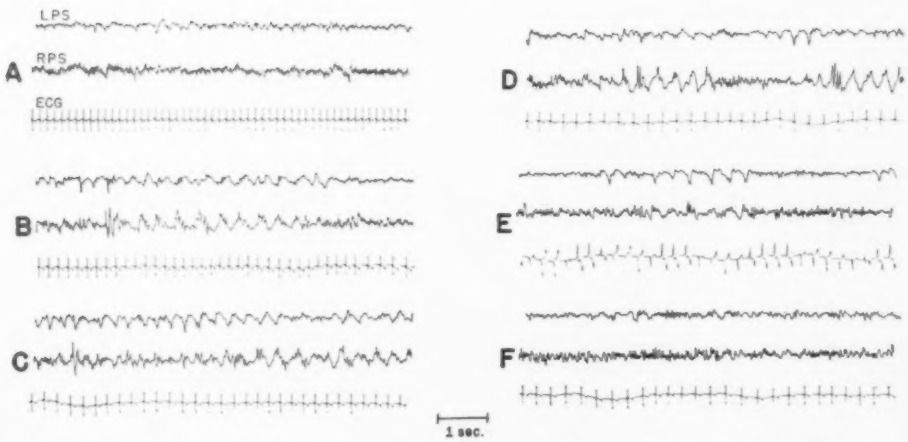

FIG. 3.—A. Electrocortical activity from *non-epileptogenic* (freezing lesion) focus (LPS); contralateral intact cortex (RPS) and electrocardiogram (ECG).
B. Changes immediately after 4 m-moles NH_4Ac.
C. Changes after 9 m-moles NH_4Ac.
D. After 15 m-moles.
E. After 17 m-moles.
F. 5 min after last injection of ammonium acetate and just before end of experiment.
Note paroxysmal activity in non-epileptogenic region and intact cortex and bradycardia (B – D) after total of 15 m-moles NH_4Ac (0·25 m-mole/kg/min). In E electrocardiographic abnormalities are profound, but reversible as shown in F.

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TABLE 2a.—CHANGES IN GLUTAMIC ACID, GLUTAMINE, GABA, AND GLUTATHIONE IN ETHYL CHLORIDE LESIONS AFTER I.V. ADMINISTRATION OF GLUTAMIC ACID

	Glutamic acid		Glutamine		GABA		GSH		Glutamic acid injected (mg/kg)	Effect of glutamic acid on focal discharges	Period between last injection and tissue removal (min)
	(μmoles/g)										
Expt.	C*	L*	C	L	C	L	C	L			
1	11·1	7·9	5·9	6·0	1·1	1·2	2·1	1·7	125	Not suppressed	25
2	9·1	8·7	5·8	6·8	1·3	2·3	2·3	2·2	100; 30 min later 100	Suppressed after 2nd injection	30
3	10·1	8·2	5·0	3·2	1·2	1·0	2·2	1·1	100; 30 min later 100	Suppressed after 2nd injection	30
4	7·8	8·9	4·4	4·2	1·2	1·4	2·0	1·4	80 for 5 doses at 5 min intervals	Transient suppression after 4 injections; suppression after last injection	5
5	8·7	6·8	5·7	5·0	1·2	1·2	2·3	1·4	80; then 50 for 6 doses at 5 min intervals	Suppressed after 1st injection	15

* C = Control, L = Lesion

administration of 100 mg of glutamine per kg revealed decreases in glutamic acid and GSH of 50 per cent and 48 per cent respectively, and normal levels of glutamine and GABA.

In studies concerned with the effects of injected ammonium acetate, attempts were made first to define the pharmacological and metabolic changes associated with slow and rapid infusions of the ammonium salt. With slow infusions (0·1–0·25 m-moles /kg/min) EEG changes consisted of intermittent periods of hypersynchronous activity that were related to slow increments in the total amount of ammonium acetate injected (Fig. 4). Recovery in such experiments was relatively rapid. With more rapid injections (0·3–0·4 m-moles/kg/min) marked but transient depression of the EEG was always observed, and subsequent recovery delayed (Fig. 5). In animals exhibiting EEG evidence of 'toxicity' brain ammonia levels of approximately 7 mg per 100 g were found, i.e. levels 20–30 times those normally found in brain.

TABLE 2b.—CHANGES IN GLUTAMIC ACID, GLUTAMINE, GABA, AND GLUTATHIONE IN ETHYL CHLORIDE LESIONS AFTER I.V. ADMINISTRATION OF GLUTAMIC ACID
(% of control values—areas contralateral to lesion)

Expt.	Glutamic acid (54)*	Glutamine (51)*	GABA (95)*	GSH (47)*
1	71	102	108	80
2	96	117	177	96
3	81	62	83	50
4	86	95	117	70
5	78	88	100	61

* % of control values in lesions before administration of glutamic acid (BERL, PURPURA, GIRADO and WAELSCH, 1959).

TABLE 3.—GLUTAMIC ACID, GLUTAMINE, GABA, GLUTATHIONE, AND ASPARTIC ACID IN ETHYL CHLORIDE LESIONS OF THE CORTEX AFTER I.V. ADMINISTRATION OF AMMONIA

	Glutamic acid		Glutamine		GABA		GSH		Aspartic acid		Ammonia		Ammonium acetate injected m-moles/kg	Type of lesion	Effect of NH_3 on lesion EEG
	(μmoles/g)														
Expt.	C*	L*	C	L	C	L	C	L	C	L	C	L			
1	7·3	4·9	5·3	4·1	1·0	1·0	1·7	1·0	2·4	1·1	0·9	0·9	1·0 in 30 min	Non-spiking	None
2	8·2	5·6	9·8	8·0	1·4	2·0	1·9	1·6	2·2	1·5	5·2	4·7	1·6 in 20 min	Non-spiking	None
3	7·5	5·2	5·6	4·1	0·7	0·9	1·7	1·0	2·4	1·3	2·5	2·5	1·7 in 7 min	Non-spiking	None
4	8·7	6·9	5·5	5·2	1·3	1·0	1·9	1·5	2·8	2·0	5·3	5·9	2·3 in 10 min	Non-spiking	Depression
5	6·5	4·6	12·2	8·7	1·2	0·9	1·8	1·0	1·2	0·9	3·2	3·9	17·5 in 70 min	Non-spiking	Complex changes (*cf.* Fig. 3)
6	7·8	5·1	5·1	6·8	1·1	1·6	1·5	1·1	3·1	1·7	0·8	1·2	1 in 30 min	Spiking	None
7	10·2	6·3	4·9	5·4	1·2	1·4	2·0	1·4	2·7	1·6	1·2	1·2	1·2 in 18 min	Spiking	None

* C = Control, L = Lesion

1–3 hr after the lesion was produced the ammonium acetate solutions (m-moles/ml) were administered intravenously in divided doses over the experimental period. The cortex was frozen *in situ* with solid CO_2 5–15 min after injection of the ammonium acetate.

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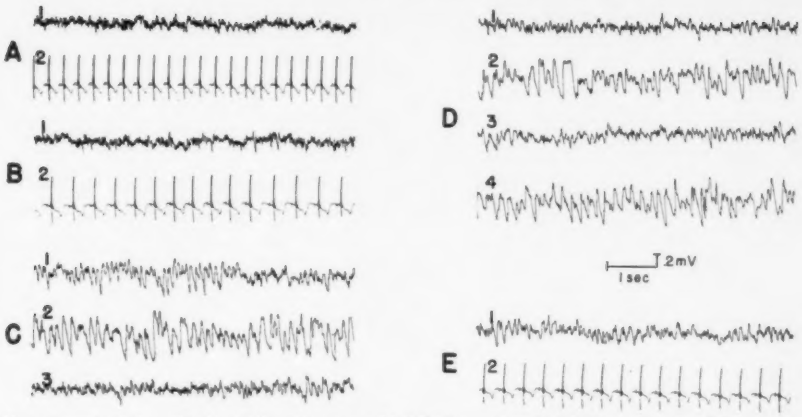

FIG. 4.—Complex effects of slow intracarotid infusion of ammonium acetate on EEG and ECG of locally anæsthetized-paralysed cat. Infusion rate 0·25 m-mole/kg/min. A. Pre-infusion EEG (1) and ECG (2). B. Same after total of 1·25 m-mole/kg of ammonium acetate. C. 1. EEG just prior to a single injection of 0·25 m-mole/kg to make a total of 2 m-moles/kg. 2. Immediately after the injection. 3. 1 min after 2. D. 1. After total 4 m-moles/kg ammonium acetate. 2. Changes just prior to injection of an additional 0·25 m-mole/kg. 3. Immediately after the additional 0·25 m-mole/kg to give a total of 4·25 m-moles/kg. 4. Few seconds after 3. E. After total 6·7 m-moles/kg in 80 min. Records taken just before end of experiment. Note tendency toward recovery of faster rhythm as in control and absence of hypersynchrony.

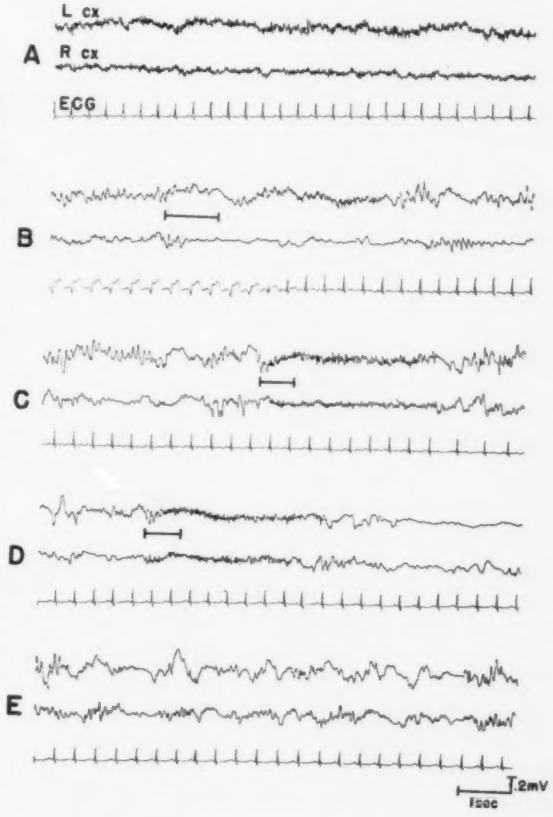

FIG. 5.—Depressant effects of rapid intracarotid infusions of ammonium acetate on EEG and ECG of locally anæsthetized-paralysed cat. A. Control records. B. 30 sec after A and after very rapid injection of 0·4 m-mole/kg ammonium acetate. Loud whistle, signalled by horizontal bar fails to alter significantly depressed electrocortical activity. Note marked cardiac irregularities. C. 1 min after B. Total of 1 m-mole/kg injected up to this point. Cardiac irregularities are minimal and 'arousal stimulus' (whistle) evokes activation pattern. D. 5 min after C, and after total of 3·3 m-moles/kg ammonium acetate. Despite marked EEG depression 'whistle' still induced electrocortical activation. E. Spontaneous activity just before end of experiment. Total amount of ammonium acetate injected, 3·3 m-moles/kg in less than 8 min.

In experimentally produced lesions, no significant increases were found in ammonia levels in the lesion sites in comparison with the contralateral control areas. Intravenously administered ammonium acetate did not initiate focal discharges in non-epileptogenic lesions, nor did ammonia augment focal discharges in epileptogenic lesions. In one short-term infusion experiment (Table 3, Expt. 4) toxic effects were observed as indicated by depression of electrocortical activity and appearance of ECG abnormalities, with relatively low concentrations. In other long-term infusion experiments EEG and ECG changes were of an extraordinarily complex nature (Fig. 3; Table 3, Expt. 5). In all experiments (Table 3) glutamic acid, GSH, and aspartic acid were decreased in lesion areas, glutamine levels were normal or increased in control and lesion sites, and GABA levels were unaltered. The fact that ammonia levels were increased in control and lesion areas clearly indicates that this metabolite effectively penetrates the series of membranes constituting the blood-brain barrier.

DISCUSSION

Considerable data are now available indicating that apart from their metabolic actions, glutamic acid and GABA exert rapid and reversible effects on a variety of excitable membranes.* The extent to which the present results permit further elucidation of the relationship between these dual actions would appear to require some knowledge of the distribution of glutamic acid and GABA in the various neural and non-neural elements in cortex. In the absence of such data, only tentative conclusions may be drawn.

Of the various relationships studied, that existing between the pharmacological action of injected GABA on low-frequency 'simple' focal discharges and the amino acid content in the lesion is the most striking. In 'untreated' lesions, glutamic acid, glutamine, and GSH are markedly decreased (BERL *et al.*, 1959). Following GABA administration, not only is the GABA content in the lesion site increased, but significant increases in glutamic acid, glutamine, and GSH are also effected. However, much of the metabolic 'recovery' in the lesion sites occurs only after several minutes, whereas the pharmacological action of GABA is detectable in seconds, i.e., in the circulation time from femoral vein to cortex.

The failure of injected GABA to alter paroxysmal activity of the 'complex' variety despite the attainment of relatively high tissue concentrations of the amino acid is of interest with respect to the postulated action of GABA on cortical synaptic organizations described in detail elsewhere (PURPURA, 1960; PURPURA *et al.*, 1959). If this action is confined to blockade of axodendritic excitatory synapses, little effect is to be expected on activities containing large components of axosomatic post-synaptic potentials as must surely be involved in the seizure activity illustrated in Fig. 1B. Thus, the differential action of GABA on the 'simple' and 'complex' paroxysmal discharges in epileptogenic lesions serves to emphasize the importance of further elucidating the relative concentrations of the amino acid at different synaptic sites, differences which are not revealed in whole cortex analyses.

The finding that intravenously administered GABA acts rapidly, whereas glutamic acid has a delayed suppressing action on focal discharges in epileptogenic lesions, suggests that the effect of glutamic acid is exerted via conversion to GABA. In

* *Inhibition of the Nervous System and γ-Aminobutyric Acid* (1960). Edited by EUGENE ROBERTS, Pergamon Press, New York.

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support of this is the finding that injected GABA behaves similarly to topical application of this amino acid while the action of systemic glutamic acid on focal discharges is in contrast to the apparently non-specific depressant effect of topically applied glutamic acid to cortex (PURPURA *et al.*, 1959). In a few instances the suppressing action of glutamic acid was preceded by a phase of augmented excitability (Fig. 2). Such differences in the topical and systemic actions of glutamic acid raise the question as to whether or not the latter may be rapidly metabolized in lesion sites before reaching the cortical neuronal surfaces. In this connection, it is of interest to note that glutamic acid levels were never increased above the normal values in lesion sites despite injections of the amino acid in high concentrations.

The pharmacological action of injected GABA occurs minutes before tissue increases are detectable. This suggests that a small compartment of GABA, at active sites, may be responsible for its pharmacological effects. Glutamine is pharmacologically inactive (PURPURA *et al.*, 1959) whereas glutamic acid and GABA exert potent pharmacological effects. These observations suggest that the interconversion of glutamic acid and glutamine may occur at a different intracellular locus from that at which glutamic acid is converted to GABA. The enzymes necessary for the former activity must be functioning since administration of ammonia produces increased levels of glutamine in the lesion as well as in normal cortex (Table 3). Evidence for compartmentalization of glutamic acid metabolism has been reported previously (LAJTHA *et al.*, 1959; TAKAGAKI *et al.*, 1961).

The altered ratio of glutamic acid to GABA in the lesion areas is of interest. One remote possibility is that the decreases in glutamic acid, glutamine, and GSH along with stable levels of GABA could occur if the former metabolites leaked out of the tissue through the traumatized 'blood-brain barrier' while GABA, more firmly fixed in tissue sites, was retained. In a previous publication (BERL *et al.*, 1959) it was suggested that in spiking lesions GABA levels are maintained at the expense of glutamic acid. However, the only described route of GABA metabolism is through transamination with α-ketoglutarate (BESSMAN, ROSSEN and LAYNE, 1953; ROBERTS and BREGOFF, 1953). This pathway establishes a feed back cycle in which for each molecule of GABA metabolized one molecule of glutamic acid, its precursor, is formed. If the loss of amino acids is metabolically produced through conversion to GABA other pathways of GABA metabolism must exist. If injected GABA reverses the drain on tissue glutamic acid this would adequately explain the observed GABA-induced increases in the levels of glutamic acid, glutamine, and GSH. Another source of glutamic acid could arise from the administered GABA by transamination with α-ketoglutarate. Part of the injected GABA, through conversion to succinic acid, would supply α-ketoglutarate by way of the citric acid cycle (ALBERS and SALVADOR, 1958).

The failure of injected ammonium acetate to alter the excitability of focal lesions in the present experiments suggests that the development of paroxysmal discharges in such traumatic lesions of cortex is not causally related to increases in tissue ammonia concentration. This is not to say, however, that infusions of ammonium acetate do not exert profound effects on the functional activity of the brain. The point of emphasis here is that with relatively slow injections of ammonia a high safety factor obtains with respect to the toxic effects of this metabolite in the unanæsthetized-paralysed cat. This is in accord with recent observations on the metabolic and

physiological effects in the central nervous system of $[^{15}N]H_4$-acetate administered by carotid infusion (TAKAGAKI *et al.*, 1961).

Apart from the demonstration that accumulation and pharmacological activity of systemically administered GABA, and to a lesser extent glutamic acid, occur preferentially in cortical regions devoid of blood-brain barrier, the present study adds little to the wealth of speculations concerning the role of GABA in brain function. If decreases in total tissue levels of GABA increase cortical 'excitability' (KILLAM and BAIN, 1957) and increases in GABA depress cortical excitability (EIDELBERG, BAXTER, ROBERTS, SOLDIAS and FRENCH, 1959), then it must be allowed that the data presented here are contrary to such notions which equate seizure susceptibility with tissue concentrations of the amino acid. These and other studies demonstrate that changes in the functional activity of cerebral cortex can occur *pari passu* with a wide variety of metabolic disturbances which are reflected, in part, in local alterations in amino acid patterns (BERL *et al.*, 1959; BAXTER and ROBERTS, 1960; PURPURA *et al.*, 1960).

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SUMMARY

The concentrations of γ-aminobutyric acid, glutamic acid, glutamine, glutathione, and in some instances aspartic acid and ammonia were determined in epileptogenic and non-epileptogenic freezing lesions of neocortex following intravenous administration of GABA, glutamic acid, glutamine, and ammonium acetate. The results were compared with the concentrations of these metabolites in intact cortex and with previously determined levels of glutamic acid, glutamine, glutathione, and GABA in lesion sites in 'untreated' preparations.

Low-frequency paroxysmal discharges were rapidly suppressed by systemically administered GABA but this immediate pharmacological action may not be reflected in changes in the levels of glutamic acid, glutamine, GSH, and GABA.

Within 15 min of systemic administration of GABA, the concentrations of glutamic acid, glutamine, and GSH tended to return to a normal level. In experiments of longer duration GABA concentrations were significantly increased in lesion sites.

Paroxysmal discharges with complex electrographic characteristics may be unaffected by systemic GABA despite dramatic increases in concentrations of the amino acid in lesion sites.

Low-frequency paroxysmal discharges were slowly depressed by multiple injections of glutamic acid. Although normal levels of glutamic acid were never attained in lesion sites, significant increases were detected in this amino acid, glutamine, and GSH.

Intravenous infusions of ammonium acetate did not produce specific changes in the excitability of epileptogenic or non-epileptogenic lesions despite significant increases in ammonia contents in these regions. Rapid infusions induced marked generalized depression of electrocortical activity, whereas slow infusions over long periods induced transient generalized bilaterally symmetrical hypersynchronous activity.

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THE BRAIN BARRIER SYSTEM—I

THE EXCHANGE OF FREE AMINO ACIDS BETWEEN PLASMA AND BRAIN

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FOLLOWING the intravenous administration of [^{14}C]-amino acids, the appearance of the label in brain within a short period of time indicates a rapid exchange of free amino acids between plasma and brain. Such rapid exchange was shown with lysine (LAJTHA, FURST and WAELSCH, 1957), glutamic acid (LAJTHA, BERL and WAELSCH, 1959), and leucine (LAJTHA, 1959), despite the fact that the net uptake of these acids from blood to brain is impeded. (For a discussion of these results see WAELSCH, 1959; WAELSCH and LAJTHA, 1961). Similar results were obtained with [^{35}S]methionine as the test amino acid (APPEL, APPEL and MAURER, 1960). From these data the average half-life of the free lysine, leucine, and methionine in the brain is estimated to be less than 1 hr.

The present report describes further studies of the exchange of amino acids between blood plasma and brain and various parts of the organ, and of the effects of altered brain amino acid concentrations on this exchange.

EXPERIMENTAL

Young male Sprague-Dawley rats (90–110 g) were used. In one type of experiment, uniformly labelled [^{14}C]-L-amino acid was injected subarachnoidally; in the other type the [^{14}C]isotope was injected intravenously and a [^{12}C] amino acid subarachnoidally. The animals were killed at various times after the injection by decapitation; the brains were removed, rinsed in ice cold 0·9% saline and frozen in dry ice. The amino acids were extracted by homogenizing three times in 5% trichloroacetic acid (TCA) and the TCA was removed on Dowex-2 columns. The amino acid content of the TCA free extract was determined microbiologically, and the radioactivity was measured in a gas flow counter. (For further details on injections, preparation of the extract, and determinations see LAJTHA, FURST, GERSTEIN and WAELSCH, 1957, and LAJTHA and TOTH, 1961).

RESULTS AND DISCUSSION

In the first group of experiments the exchange of amino acid between plasma and brain was studied after the amino acid concentration in brain had been increased several-fold. Following increase in brain level by subarachnoid [^{14}C]amino acid injection, maximal loss of radioactivity from this organ would occur if the injected amino acid did not mix with the amino acid already present and all the subsequent loss occurred from this separate labelled pool. The specific activity would decrease with time and become zero when the control amino acid level was reached. Minimal loss would occur if the injected amino acid did not mix and the loss subsequently occurred not from the administered labelled amino acid but from the unlabelled amino acid

pool originally present in the brain. The specific activity in the brain would increase till it approximated that of the administered amino acid.

The experiments were designed to decide between these alternatives, since empirically determined specific activities can be compared with theoretical values. From cerebral amino acid concentration increase at a given time, and from the specific activity of the injected amino acid, upper and lower limits of expected radioactivity in the brain can be calculated if other factors such as the metabolism of the amino acids are neglected.

Measured values for specific activities of amino acids at various times after intracerebral injections are given as well as the values calculated by assuming maximal and minimal losses in radioactivity (Table 1). For the initial phase of the experiment,

TABLE 1.—CHANGES IN SPECIFIC ACTIVITIES OF AMINO ACIDS IN RAT BRAIN AFTER THEIR SUBARACHNOIDAL ADMINISTRATION

Time (min)	Lysine		Leucine		Phenylalanine		Proline	
	Calculated*	Found	Calculated*	Found	Calculated*	Found	Calculated*	Found
1	110	110	210	200	230	240	190	170
5	160	150	220	170	230	230	190	190
20	220	160	160–275	110	230	230	200	210
45	220	140	46–275	28	210–250	160	160–240	210
120	200–260	45	46–275	19	140–250	83	0–240	140
180	180–300	24	0–275		0–250			
240	140–300	10						

* Specific activity calculated for no mixing of the 'injected' amino acid with the 'existing' pool. For minimal specific activity the loss postulated to occur only from the pool of the 'injected' amino acid; conversely for maximal specific activity it is postulated that the initial loss occurs from the 'existing pool'. The specific activities of the injected amino acids were: lysine: 300; leucine: 275; phenylalanine: 250; and proline: 240 counts/min/μg. 0·02 ml of one of the following amino acids was injected subarachnoidally into young male rats: lysine: 14 mg/ml; leucine: 14 mg/ml; phenylalanine: 18 mg/ml; proline: 12 mg/ml. Average of four experiments.

when the amino acid concentration is increasing in the brain, the assumption is made that there is no loss in radioactivity; therefore, the maximal and minimal values coincide and only one value is presented. Most of the measured values are below the calculated minimal values, a finding which indicates that other factors are operative in diluting the specific activity of the amino acid in the brain. It is not likely that significant dilution occurred because of protein turnover or because of the metabolic turnover of the amino acid within the brain in these short-time experiments. The decrease in specific activity appears to be due to the exchange of labelled cerebral amino acid with the unlabelled plasma amino acid. The rapid exchange of amino acids between the plasma and brain thus must occur even when the amino acid in the brain is increased above normal and there is a net efflux of brain amino acid during the experiment. In many cases the measured specific activity was below the calculated one in the initial phase of the experiment while the amino acid was being increased. This shows that exchange occurs also while there is a net influx of the amino acid. Without the knowledge of the extent of mixing in the brain and of the true specific activity of the amino acid being transported out of the brain, the rate of the exchange

reaction cannot be accurately measured, but in magnitude it seems to be similar to that measured previously without additional intracerebrally administered amino acids.

The exchange between brain and plasma amino acids was further investigated by comparing exchange rates in brains with and without increased amino acid levels. In these experiments all animals were injected intravenously with tracer doses of labelled amino acids without significantly increasing the concentration of the amino acid in the plasma, and the appearance of the labelled amino acid in the brain (by exchange) was measured. In half of these animals, brain amino acid level was increased by subarachnoid injection of unlabelled amino acid. The finding (Table 2) that the

TABLE 2.—RADIOACTIVITY IN PLASMA AND BRAIN AFTER INTRAVENOUS ADMINISTRATION OF [^{14}C]AMINO ACID

Time (min)	Injection	Lysine			Leucine		
		Plasma (counts/g/min)	Brain (counts/g/min)	$\frac{\text{Plasma}}{\text{Brain}}$ ratio	Plasma (counts/g/min)	Brain (counts/g/min)	$\frac{\text{Plasma}}{\text{Brain}}$ ratio
5	Intravenous [^{14}C]	62,000	6900	9·0 ± 1·0	19,000	8800	2·2 ± 0·3
5	Intravenous [^{14}C] and subarachnoidal [^{12}C]	58,000	11,000	5·5 ± 0·4	16,000	14,000	1·2 ± 0·2
10	Intravenous [^{14}C]	35,000	8100	4·3 ± 0·8	13,000	9500	1·4 ± 0·2
10	Intravenous [^{14}C] and subarachnoidal [^{12}C]	32,000	14,000	2·3 ± 0·2	12,000	16,000	0·8 ± 0·05

Intravenous injections: 0·1 ml of an amino acid solution containing 50 μc/ml and 5 μc/μmole at 1 min.
Subarachnoidal injections: 0·02 ml of a 14 mg/ml solution at the beginning of the experiment.
Young male rats used, one animal for each experiment.
Average of three experiments.

ratio of radioactivity per gram of blood plasma to radioactivity per gram of brain, a measure of exchange, is less in brains with high amino acid levels indicated an increased rate of exchange when brain levels are increased. The increase in exchange rate occurred after either lysine or leucine administration.

It was of importance to find out whether the subarachnoidally administered amino acid is present in the spinal fluid adhering to the organ, or in the superficial layers of the brain, or is evenly distributed throughout the brain. To a group of rats, [^{14}C]-lysine or leucine was administered subarachnoidally, and the animals were killed 5 min later. The brain was removed and rinsed with cold 0·9% saline. The cerebellum, pons, and medulla were discarded and the cerebrum was frozen in dry ice and sectioned horizontally into seven parts. The lateral and frontal cortex were added to the top (first section) so that only the first and seventh sections contained cortex. The sections were of about the same weight. The distribution of label in each section (Table 3) shows that the injected amino acid is present in the deeper layers of the brain as well as in the superficial ones.

The finding of an increased amount of radioactivity in the free amino acids of the brain after its amino acid concentration is increased might also be due to the fact that the incorporation of labelled amino acid into proteins (therefore loss from the free amino acid pool) will cause a smaller decrease because of the dilution of the specific activity of the free amino acid in brain by the intracerebrally injected non-labelled acid.

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TABLE 3.—PENETRATION OF SUBARACHNOIDALLY ADMINISTERED [^{14}C] AMINO ACIDS INTO THE BRAIN

Brain section		Relative distribution (counts/g brain whole cerebrum = 100)	
		Leucine	Lysine
1	Superior	23	16
2		53	83
3		100	140
4		180	180
5		120	110
6		94	88
7	Inferior	98	99
Counts/g whole cerebrum		11,000	63,000

Percentage of the injected dose in brain: leucine 21%, lysine 19%.
Percentage of the injected dose in the saline wash fluid: leucine, lysine 4%.
2 μmole of the amino acid injected subarachnoidally in 0·02 ml, 5 min experiment. Single rats used for each experiment; average of three experiments.

If protein turnover proceeds at the same rate in intracerebrally injected as in control animals, the same amount of incorporation of amino acid with a lower specific activity will result in a smaller loss of radioactivity in the free amino acid pool. That the results reported in Table 2 are not due to the differences in incorporation into proteins is shown in Table 4. Although more radioactivity was incorporated into the proteins in brains in which the free amino acid pool was not diluted, the sum of radioactivity in protein bound and free amino acid was greater in brains with increased amino acid levels. This result shows that an increase in exchange rate is of major influence whereas changes in incorporation are not.

The effect of subarachnoidal injection of amino acids and NaCl on the plasma-brain exchange of amino acids injected intravenously was investigated (Table 5). The effect of physiological saline was variable—slightly increasing lysine exchange and decreasing leucine exchange. When compared to the saline injection, amino acid injection (to increase the amino acid level in the brain) caused increased exchange,

TABLE 4.—DISTRIBUTION OF [^{14}C] AMINO ACID IN RAT BRAIN 10 MIN AFTER INTRAVENOUS ADMINISTRATION

Injection		[^{14}C] (counts/min/g brain tissue)		
Intravenous	Intracerebral	In protein	In free amino acid	Total
[^{14}C] lysine	none	2200	11,000	13,000
[^{14}C] lysine	[^{12}C] lysine	1600	18,000	20,000
[^{14}C] leucine	none	12,000	12,000	24,000
[^{14}C] leucine	[^{12}C] leucine	9000	26,000	35,000

For experimental details see Table 2. Average of two experiments.

TABLE 5.—THE EFFECT OF SUBARACHNOID INJECTIONS ON THE EXCHANGE RATE OF AMINO ACIDS BETWEEN PLASMA AND BRAIN

Injection		Counts/g fresh brain/min	Change % in comparison with:	
Intravenous	Subarachnoidal		No subarachnoid injection	NaCl injection
Lysine	—	3300 ± 660		
	Lysine	4800 ± 710	+45	+26
	Leucine	4300 ± 550	+30	+13
	NaCl	3800 ± 690	+15	
Leucine	—	5500 + 610		
	Lysine	5500 ± 740	0	+34
	Leucine	7100 ± 820	+29	+73
	NaCl	4100 ± 510	−25	

Young male rats were injected subarachnoidally, then intravenously 1 min later and were killed 5 min after the intravenous injection.

Intravenous injection: [^{14}C]lysine or [^{14}C]leucine 0·02 ml of a solution containing 0·2 μmole ($1{\cdot}2 \times 10^6$ counts/min).

Subarachnoid injection: 0·02 ml of 0·9% saline or 0·9% saline containing 1·9 μmole [^{12}C]lysine or 2·1 μmole [^{12}C]leucine.

Average of four experiments.

lysine having a greater effect on lysine exchange than had leucine and conversely leucine having a greater effect on leucine exchange than had lysine. Cerebral amino acids thus exert an influence on their own exchange rates. Exchange processes are probably a part of the mechanism responsible for regulation of the composition of the brain. The refinement of this regulation can be seen from the fact that a distinction between individual amino acids can be made and the exchange rate of one amino acid can be increased over the exchange rate of another. It is likely that such changes in uptake or exchange rates can follow changes in metabolism or of metabolite concentrations in the brain.

To ascertain if the exchange occurs throughout the brain or only in specially localized areas, the distribution of amino acids in six brain areas of control rats was determined (Table 6) and compared with the distribution of radioactivity in rat brain

TABLE 6.—DISTRIBUTION OF AMINO ACIDS IN BRAIN PARTS

	Amino acid (μg/g fresh brain)		
	Lysine	Leucine	Phenylalanine
Cerebellum	64 ± 3	17 ± 2	17 ± 3
Pons-medulla	78 ± 8	14 ± 2	13 ± 2
Posterior superior cerebrum	42 ± 5	14 ± 2	15 ± 2
Posterior inferior cerebrum	52 ± 7	15 ± 3	17 ± 3
Anterior superior cerebrum	34 ± 4	15 ± 3	11 ± 2
Anterior inferior cerebrum	39 ± 5	16 ± 2	16 ± 3
Whole brain	49 ± 4	15 ± 2	15 ± 3

Ten young rat brains were sectioned into six parts for each set of determinations. Results show the average of three experiments.

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after the intravenous injection of tracer doses of [^{14}C] amino acids. The amino acid levels were not uniform throughout the brain.

The relative distribution of [^{14}C]lysine is shown in Table 7. In the control rat a somewhat higher lysine content was found in the cerebellum and in the pons-medulla and a lower content in the anterior part of the brain. After intravenous administration of tracer doses of [^{14}C]lysine more label appeared in areas that had a relatively higher content and less label in areas with lower content; this points to an increased exchange rate in areas of relatively higher levels. The rate of exchange in normal brains thus is not the same throughout the organ but may vary from one area to another. After subarachnoid injection of L-lysine the absolute brain concentration is doubled but the

TABLE 7.—RELATIVE DISTRIBUTION OF LYSINE IN RAT BRAIN PARTS

	L-Lysine				D-Lysine
	Control* (calculated from Table 6)	Intravenous†	Subarachnoid*	Intravenous and subarachnoid†	Subarachnoid†
Cerebellum	130	130	140	130	140
Pons-Medulla	160	140	120	130	130
Posterior superior cerebrum	86	110	110	98	110
Posterior inferior cerebrum	110	87	120	93	130
Anterior superior cerebrum	67	83	60	88	44
Anterior inferior cerebrum	78	75	78	94	75

Whole brain = 100. Young (90–110 g) male rats killed 5 min after intravenous amino acid administration.

* μmole lysine/g fresh brain.

† Counts/min/g fresh brain.

Intravenous injection: 0·2 ml and 8 μc and 1 μmole [^{14}C]-L-lysine solution.

Subarachnoid injection: 0·02 ml and 1·9 μmole of [^{12}C]-L-lysine.

D-Lysine injection: 0·02 ml, 1·9 μmole and 0·02 μc [^{14}C]-D-lysine subarachnoidally.

The intravenous injection followed the subarachnoid by 1 min. Average of three experiments.

relative distribution of lysine is similar to that in control animals, relatively more lysine being taken up in areas that normally had higher levels and less in areas with lower levels. D-Lysine after its subarachnoid administration is distributed similarly to the L form. The relative distribution after brain lysine levels were increased by subarachnoid injection of [^{12}C] amino acid and by intravenous injection of [^{14}C]lysine is also shown. The appearance of label again is greater in areas with relatively higher concentrations; this shows that unequal levels in the various areas result in unequal exchange rates whether the absolute level is increased or is at control level.

It has to be emphasized that, although in general higher lysine level in a brain area is followed by increased exchange rate, this relationship is by no means a very strict one. A clearer picture will result if the exchange rates of a compound that shows a much more pronounced difference in level from one brain area to another are measured. Uneven distribution of some amino acids in various brain areas has been shown (BERL and WAELSCH, 1959; OKUMURA, OTSUKI and FUKAI, 1959; and PRICE and WEST, 1960) but the differences described were not very great.

To show clear differences in exchange rates, it is necessary to use a compound with

a marked uneven distribution in brain areas. When leucine was used as the test amino acid (Table 8) the levels in control rats were fairly uniform throughout the brain, much more so than was lysine, and the exchange rates with and without increased brain leucine levels did not differ significantly from one brain area to another.

The results discussed in this paper show that the exchange rates of substrates are not necessarily constant. The exchange rate of one amino acid can be increased without a concomitant increase in the exchange rate of other amino acids; also, exchange rates may vary from one brain area to another.

TABLE 8.—RELATIVE DISTRIBUTION OF LEUCINE IN RAT BRAIN PARTS

	Control* (calculated from Table 6)	Intravenous†	Subarachnoid*	Intravenous and subarachnoid†
Cerebellum	110	120	140	110
Pons-Medulla	90	85	110	97
Posterior superior cerebrum	95	94	140	93
Posterior inferior cerebrum	100	96	100	100
Anterior superior cerebrum	100	100	58	88
Anterior inferior cerebrum	100	110	88	110

Whole brain = 100. Experimental procedure similar to that described in Table 7 with leucine replacing lysine.
* μmole leucine/g fresh brain.
† Counts/min/g fresh brain.
Average of three experiments.

Under physiological circumstances, alterations of influx or efflux rates might serve as a homoeostatic mechanism to restore rapidly any change in brain composition or to supply at varying rates the changing metabolic needs of the brain. The different exchange rates at the various brain areas might indicate the differences in need for the metabolites in these areas.

SUMMARY

Following intravenous administration of tracer doses of [^{14}C] amino acid the label appears in brain quickly, showing a rapid exchange between plasma and brain. This exchange also occurs when brain amino acid levels are increased and there is a net influx into or efflux of amino acids from the brain with time.

The exchange rates of amino acids are increased by increased brain amino acid concentrations with a particular amino acid increasing its own exchange more than it increases the exchange of other amino acids.

The exchange of plasma lysine with that of brain lysine seems to occur at various rates throughout the brain and to some extent is proportional to the concentration of the amino acid in the brain part investigated. The significance of the differences established by measuring the exchange of leucine in various brain parts is questionable.

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THE DISTRIBUTION OF CATIONS IN PLASMA AND CEREBROSPINAL FLUID FOLLOWING INFUSION OF SOLUTIONS OF SALTS OF SODIUM, POTASSIUM, MAGNESIUM AND CALCIUM

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EARLIER experiments, in which the concentration of Mg in the plasma of anaesthetized dogs was maintained at a high level for 5 to 24 hrs, have shown that the Mg^{++} content of cerebrospinal fluid is independent of the plasma level. This suggests that the stability of the Mg concentration in the CSF is maintained by a selective mechanism involving active transport of this ion (KEMÉNY, BOLDIZSÁR and PETHES, 1961). The present report provides confirmation of this idea and also considers the distribution of other cations.

Two types of experiment were undertaken. In the first (Type 1, Table 1) the concentration of Mg^{++} in the plasma was kept artificially high for several hours by means of an intravenous infusion, the water content of which brought about a decrease in the concentration of other plasma cations. In the second type of experiment (Type 2, Table 1), a simultaneous rise in plasma K^{+} and plasma Ca^{++} as well as plasma Mg^{++} were induced and the effect on the corresponding concentrations in CSF was determined. Experiments of the second type were also carried out in the presence of low plasma sodium concentrations.

METHODS

Experimental conditions

Acute experiments were performed on 31 mongrel dogs and bitches weighing about 15 kg, under chloralose narcosis. The experimental arrangements were similar to those previously described (KEMÉNY, BOLDIZSÁR and PETHES, 1961). Two types of infusion were used; details of their composition are given in Table 1.

In the second type of infusion experiment, $MgSO_4$ solution was infused from a burette inserted into one femoral vein and $CaCl_2$ and KCl solutions were infused into the other of a dog lying on its side. Blood samples were obtained from the jugular vein and CSF samples from the cisterna magna. When CSF pressure was continuously registered, samples were taken by means of a T-shaped branch-pipe.

Heart rate, respiration and arterial pressure were continuously registered; venous pressure in the jugular vein and CSF pressure in the cisterna were monitored several times, using the method described previously. Temporary irregularities of the heart rate or fluctuation of blood pressure could be, for the most part, compensated for or normalized by infusions of solutions of antagonistic ions or by interrupting the infusion for a few minutes. Hypermagnesaemia led in some cases to respiratory paralysis and a breathing apparatus had to be applied. At the time of taking the last samples, circulation generally proved to be in the physiological range. The data published in this paper are only from animals whose circulation did not deteriorate in the course of the experiment. Characteristic changes were as follows.

TABLE 1.—DETAILS OF THE TWO TYPES OF INFUSION USED IN THE EXPERIMENTS

Type of experiment	Solution infused	Rate of infusion (mg cation/kg body wt per hr)	Volume infused (ml)	Time of infusion (hr)
1	1% $MgSO_4$ (in H_2O)	12–48	700–1000	5
2	2% KCl 2% $CaCl_2$ 1% $MgSO_4$ (in 0·45% NaCl)	80–120 40–60 12–48	140–550 150–450 410–960	5 (4·6·5)

In hypermagnesaemia the arterial pressure of dogs narcotized at the beginning of the experiment only fell from 110/95 mm Hg to 90/83 mm Hg by the end of the experiment (mean values of 7 animals); venous pressure rose from 75 to 88 mm H_2O and the CSF pressure rose from 65 to 72 mm H_2O (mean values of 5 animals). In experiments in which simultaneous infusions of salt solutions were used arterial pressure fell from 130/120 mm Hg to 105/100 mm H_2O (mean values of 4 animals); CSF pressure fell from 48 to 35 mm H_2O (mean values of 5 animals). In two control experiments 0·9% NaCl solution was infused. The changes in arterial, venous, and CSF pressures were similar to those obtained with simultaneous infusions of salt solutions. Normal body-temperature was ensured throughout the experiment by warming the animal. At the end of the experiment the animal was killed.

Chemical analysis

Potassium and sodium were determined by flame photometry, calcium according to the method of CRAMER and TISDALL modified by CLARK and COLLIP (1925), magnesium was determined by the titan yellow method of ORANGE and RHEIN (1951) modified by BOLDIZSÁR and PETHES (1959).

All results given in the text and in the tables are means ± standard error of the mean. The significance of differences between means was estimated by Student's '*t*' test and results are given as the probability (*P*) value obtained.

RESULTS

Effect of infusions of $MgSO_4$ *solution on the cation composition of the CSF*

Altogether 21 dogs have been used for this study. Samples of blood (10 ml) were withdrawn every 0·5–1 hr to control the level of plasma Mg. The results of determinations on 16 of the dogs are shown in Table 2. Samples of CSF were taken directly before starting the infusion in order to establish the initial cationic composition. From a further 5 dogs a CSF sample was taken at the end of the experiment only, viz. at the 5–6 hr of the infusion, in order to determine whether or not the withdrawal of CSF at the beginning of the experiment influences the results obtained.

Although the concentration of Mg^{++} in the plasma was increased considerably above the normal value, the CSF values rose only to the upper limit of the normal range. This is in agreement with our earlier findings (KEMÉNY, BOLDIZSÁR and PETHES, 1958). The concentration of K^+ and Ca^{++} showed a significant decrease in the plasma and in the CSF, although this fall was small. Similar values of K^+ and Ca^{++} (Table 3) were obtained in the 5 animals in which a CSF sample was taken at the end of the infusion; thus showing that the removal of a sample of CSF initially did not affect the results.

Normally the concentration of sodium in the plasma somewhat exceeds that in the CSF. By the end of the experiment the plasma sodium concentration had decreased and the CSF concentration of sodium had increased. If the data are corrected for water content the concentration of sodium in the CSF is still lower than that of plasma but the difference is reduced. Comparing the initial and final values obtained for CSF sodium concentration, it appears that in the course of the experiment the concentration of sodium exhibits a rising tendency, the changes, however, are not significant. The changes in the CSF sodium concentration, obviously due to scatter within the groups, were not significant presumably in connection with the fact that the plasma sodium levels varied according to the loads administered in the course of the experiment.

The changes in estimated concentration in the CSF of the four cations may, to some extent, reflect the altered distribution of water between plasma and CSF space. The haematocrit values indicate a slight dilution in the plasma. (In 4 animals the

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TABLE 2.—DISTRIBUTION OF CATIONS BETWEEN BLOOD AND CSF AFTER INFUSIONS OF $MgSO_4$ SOLUTION

	Na		K		Ca		Mg	
	Pl	CSF	Pl	CSF	Pl	CSF	Pl	CSF
Beginning of experiment								
m-equiv./l.	153·6 ± 1·5	151·3 ± 1·2	5·50 ± 0·20	4·10 ± 0·15	5·10 ± 0·20	3·20 ± 0·23	1·50 ± 0·09	1·58 ± 0·07
m-equiv./H_2O kg*	167·0	152·8	5·98	4·14	5·53	3·23	1·63	1·60
End of experiment								
m-equiv./l.	147·8 ± 0·7	157·6 ± 0·7	4·85 ± 0·50	3·92 ± 0·07	4·65 ± 0·49	2·85 ± 0·18	10·20 (6·3–24·5)	1·95 ± 0·35
m-equiv./H_2O kg*	160·6	159·2	5·27	3·95	5·05	2·87	11·10 (5·2–12·4)	1·97
		$0·05 < P < 0·1$		$P < 0·02$		$P < 0·02$		$P < 0·01$
No of animals	7		7		10		16	

* Assuming a water content in plasma of 92 per cent and in CSF of 99 per cent.

TABLE 3.—DATA FOR 5 ANIMALS

	K^+		Ca^{++}	
	m-equiv./l.			
	Beginning of expt.	End of expt.	Beginning of expt.	End of expt.
Plasma	5·2 ± 0·3	4·7 ± 0·2	5·3 ± 0·4	4·2 ± 0·4
CSF	4·1 ± 0·1	4·0 ± 0·1	3·2 ± 0·2	2·8 ± 0·3

haematocrit values decreased from the initial mean value of 44·5 to 42·7 by the end of the experiment. In control experiments using infusions of physiological NaCl solution, the decrease was from 44 to 38·5). The changes in potassium and calcium concentration in the CSF may be attributed partly to dilution, i.e. to the movement of plasma water towards CSF space. However, since the concentration of the four CSF cations measured did not change in the same direction, the alterations in level of potassium and calcium may be also explained by diminished saturation of supposed carrier substances in the structure of the barrier.

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The joint effects of hypermagnesaemia, hypercalcaemia, hyperkalaemia on the distribution of CSF cations, varied with hyper- and hyponatraemia

The results of one experiment (dog no. 147) are given in full (Table 4). The $MgSO_4$, KCl and $CaCl_2$ used for infusions were dissolved as a rule in 0·45% NaCl solution, but in some cases (e.g. in that of dog no. 147) in 0·9% NaCl. In two experiments hyposodaemia was produced by using distilled water as solvent. In order to show the steady rise in plasma level, plasma samples were withdrawn at intervals and analysed while CSF values were determined only at the end of the experiment. Our results are shown in Table 5.

As can be seen, the concentrations reached and the duration of the elevated plasma level ensures favourable conditions for studying the behaviour of the membrane separating plasma-space from CSF-space, i.e. prolonged overloading is achieved in the course of the experiment.

In the experiments recorded in Table 5 the solutions infused were slightly hypertonic, and when the total levels of cations in plasma and CSF are considered it can be seen that there was a surplus of osmotically active solute in the plasma at both the beginning and end of the experiments: this is so whether the concentrations are expressed per litre of solution or per kg of water. In the case of sodium, when the concentrations are expressed per unit weight of water the plasma/CSF difference disappears at the end of the experiment suggesting that equalization has taken place by a passive process. The change of concentration of sodium during the course of the experiments (from 151 to 158 m-equiv./kg water) which was highly significant statistically ($P < 0·01$) might thus be explained by shifts of the distribution of water. However, the other cations studied did not all show changes in the same direction or of the same magnitude. Thus the distribution ratio of potassium decreased during the experiments from 0·61 to 0·45 while for calcium the ratio altered from 0·48 to 0·14, the concentration in the CSF being very little changed during the course of the experiment. Lowering of the plasma sodium level by some 40 m-equiv./l. in 2 animals

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TABLE 4.—DISTRIBUTION OF CATIONS BETWEEN PLASMA AND CSF AFTER SIMULTANEOUS I.V. INFUSIONS OF MIXED SALINE SOLUTIONS
(Dog no. 147, 20 kg, male. Narcotized: 100 ml 1% chloralose intravenously, received 210 ml 5% $MgSO_4$ subcutaneously)

	Time of taking samples, from the beginning of the experiment (hr)	Na		K		Ca		Mg	
		Pl	CSF	Pl	CSF	Pl	CSF	Pl	CSF
m-equiv./l. m-equiv./H_2O kg*	0	150·9 164·0		5·90 6·41		5·00 5·43		1·50 1·60	
m-equiv./l. m-equiv./H_2O kg*	2·5	155·2 168·7		6·05 6·58		8·20 8·91		10·20 11·10	
m-equiv./l. m-equiv./H_2O kg*	5·0	147·8 160·6		8·97 9·75		12·50 13·60		17·30 18·80	
m-equiv./l. m-equiv./H_2O kg*	6·5	157·8 171·5	170·0 171·7	7·60 8·20	5·55	15·70 17·10	3·43	20·40 22·11	2·52
Solutions†		in 0·9% NaCl‡		550 ml 2% KCl		450 ml 2% $CaCl_2$		750 ml 1% $MgSO_4$	

* The assumed water content was 92 per cent in plasma and 99 per cent in CSF.
† Total volume of liquids: 2060 ml.
‡ In this experiment the plasma Na concentration was somewhat increased.

TABLE 5.—DISTRIBUTION OF CATIONS BETWEEN PLASMA AND CSF
(Mean values

	Na			K			Ca		
	Pl	Ratio	CSF	Pl	Ratio	CSF	Pl	Ratio	CSF
Beginning of experiment									
m-equiv./l.	151·1 ± 1·2	0·98	149·5 ± 0·6	5·85 ± 0·10	0·66	3·88 ± 0·20	6·05 ± 0·30	0·51	3·10 ± 0·10
m-equiv./H_2O kg*	164·2	0·94	151·0	6·36	0·61	3·92	6·57	0·48	3·13
End of experiment									
m-equiv./l.	144·8 ± 0·9	1·08	156·5 ± 1·3	10·22	0·44	4·92 ± 0·50	19·25	0·15	2·90 ± 0·30
m-equiv./H_2O kg*	157·4	1·00	158·1	11·11	0·45	4·97	20·93	0·14	2·93
Significance			$P < 0·01$			$0·1 > P > 0·05$			$P > 0·05$

* Assumed water content of plasma 92 per cent and CSF 99 per cent.

did not materially affect the plasma CSF ratios of K^+, Ca^{++} and Mg^{++} following infusion of their salts (Table 6).

DISCUSSION

In addition to shedding light on the qualitative nature of the blood–CSF barrier, we consider that our loading tests may provide a basis for thermodynamic calculations on the chemical work done in producing CSF.

Somewhat similar experiments have been reported by CAMERON and MOORHOUSE (1937) for Ca^{++}, and by BEKAERT and DEMEESTER (1951) for K^+. However, these workers, apart from investigating only one ion at one time, dealt only with relatively slight changes in concentration. The older experiments of STERN and RAPOPORT (1927) in which various substances were introduced into the blood stream are relevant but difficult to interpret precisely. COOPER, LEXNER and BELLET (1955) found that the potassium level in CSF is relatively independent of the plasma level; no similar independence was found in the case of sodium. The interpretation of their findings is complicated by the fact that many of their animals died in a matter of 12 min from the administration of potassium, while their results are expressed without correction for other dissolved materials.

In our opinion the CSF space may be compared to a huge cell vacuola, the composition of which is determined partly by the special biochemical characteristics of the cells and partly by active cell function. In the light of this, can the CSF be regarded as 'Totes Füllmaterial' as LANGE (1935) supposed it to be? Among the anatomical structures producing the CSF, it is only the choroid plexus to which such cell function could be attributed. KROGH's (1946) statement that the blood–brain barrier resembles a cell-membrane could be extended to the blood–CSF

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AFTER SIMULTANEOUS I.V. INFUSIONS OF SALINE SOLUTIONS
for 8 dogs)

Mg			Total cations		Osmotic pressure of cations (m-osmoles)	
Pl	Ratio	CSF	Pl	CSF	Pl	CSF
1·58 ± 0·17	1·00	1·58 ± 0·25	164·58	158·06	per l. 160·8	156·8
1·72	0·93	1·60	178·90	159·70	per 174·6 kg H_2O	157·3
16·30	0·13	2·17 ± 0·40	190·60	166·50	per l. 172·7	164·0
17·70	0·12	2·20	207·10	168·20	per 187·8 kg H_2O	165·7
		$P < 0·01$				

barrier. The observed responses to Ca^{++} and Mg^{++} and probably also to K^{+} may, be best explained by secretion in which carrier substances play a role. The other theoretical possibility, the existence of a mechanism for filtration and reabsorption may be almost certainly excluded on the grounds that CSF calcium and magnesium levels were completely independent of the varying plasma levels.

A number of works which have appeared recently in connection with CSF have involved the use of tracer techniques. Since the pioneer studies of GREENBERG and his collaborators (GREENBERG *et al.*, 1943) valuable information has been acquired regarding the kinetics of ion-exchange between plasma and CSF. But tracer techniques have their limits and in discussing them SELVERSTONE (1958) emphasises that ion-exchange occurs across the barrier when a steady state is maintained on a 1:1 basis, in which case we do not get information about its biological significance but only on the permeability of the membrane. In this system, thermodynamically no work is being done. We may add that information is also scarce regarding the biological function of ions since the *in vivo* determination of CSF volume is not readily practicable (FISHMANN, 1959) and this is a grave disadvantage in examinations carried out either by straightforward chemical methods or by tracer techniques.

SUMMARY

The distribution of cations between plasma and CSF space was studied in 31 dogs after i.v. infusion of salt solutions lasting 5–6 hr.

On raising the concentration of Mg^{++} in plasma to from 6 to 24 m-equiv./l. by infusion of Mg salts, the concentration of Mg^{++} in the CSF did not change.

On producing hypercalcaemia, hyperkalaemia and hypermagnesaemia, the

TABLE 6.—DISTRIBUTION OF CATIONS BETWEEN PLASMA AND CSF AFTER SIMULTANEOUS INFUSIONS OF SALINE SOLUTIONS DURING HYPONATRAEMIA

	Na		K		Ca		Mg		Total cations	
Beginning of experiment	Pl	CSF	Pl	CSF	Pl	CSF	Pl	CSF	Pl	CSF
m-equiv./l.	144·0	–	6·10	–	6·25	3·60	1·90	–	158·30	–
m-equiv./H_2O kg	156·5	–	6·63	–	6·80	3·64	2·07	–	172·00	–
End of experiment										
m-equiv. /l.	105·0	115·0	7·30	4·30	17·30	2·90	9·10	2·00	138·70	124·20
m-equiv./H_2O kg	114·0	116·5	7·94	4·34	18·80	2·93	9·90	2·02	150·50	125·40

Two animals used.

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concentration of Ca, K and Mg ions in the CSF remained unchanged. The concentration of Na rose (from 151 to 158 m-equiv./kg water), although the plasma values remained unchanged. The characteristic features of the distribution of Na are discussed.

The findings suggest the existence of a selective mechanism ensuring the stability of the level of cations in the CSF. The participation of carrier substances has been postulated.

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MICRODETERMINATION OF URIDINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE IN BRAIN*

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THE finding that glycogen is synthesized from uridine diphosphate glucose (UDP-glucose§) in animal tissues (LELOIR *et al.*, 1957, 1959, 1960) including brain (BRECKENRIDGE and CRAWFORD, 1959, 1960) has focused interest upon UDP-glucose pyrophosphorylase, which catalyses the formation of UDP-glucose from glucose-1-phosphate (glucose-1-P) and uridine triphosphate (UTP) (Equation 1).

$$\text{UTP} + \text{glucose-1-P} \rightleftharpoons \text{UDP-glucose} + \text{inorganic pyrophosphate} \qquad (1)$$

While the occurrence of the UDP-glucose pyrophosphorylase reaction has been noted in brain (MAXWELL, KALCKAR, and BURTON, 1955), optimal conditions for its measurement in that tissue have not been determined previously. The enzyme has been purified from yeast (MUNCH-PETERSEN, 1955), mung beans (GINSBURG, 1958), and rabbit muscle (VILLAR-PALASI and LARNER, 1960*a*). The present paper describes a new micro-assay and the optimal conditions for measuring UDP-glucose pyrophosphorylase activity in brain. UDP-glucose formed in the pyrophosphorylase reaction (Equation 1) is measured by the reduction of diphosphopyridine nucleotide (DPN^+) in the presence of UDP-glucose dehydrogenase (Equation 2).

$$\text{UDP-glucose} + 2DPN^+ \rightarrow \text{UDP-glucuronate} + 2DPNH + 2H^+ \qquad (2)$$

The assay is both sensitive and convenient and may be especially useful for investigating certain problems mentioned in the discussion.

MATERIALS

Glucose-1-P, UTP, and DPN^+ were obtained from the Sigma Chemical Company. UDP-glucose dehydrogenase was prepared by the method of STROMINGER, MAXWELL, AXELROD, and KALCKAR (1957). The enzyme preparation ordinarily used was carried through Step 5 (precipitation with $(NH_4)_2SO_4$ between 35% and 55% saturation at pH 8) and aged for at least 2 months at pH 5·9 and −20° to remove traces of UDP-glucose pyrophosphorylase. The enzyme was concentrated by reprecipitation with $(NH_4)_2SO_4$. The final preparation contained 21,000 units per ml (640 units per mg protein) and had no activity with components of the system other than UDP-glucose.

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§ The abbreviations used are: UDP-glucose, uridine diphosphate glucose; glucose-1-P, glucose-1-phosphate; UTP, uridine triphosphate; DPN^+, diphosphopyridine nucleotide.

Measurement of UDP-glucose pyrophosphorylase. The first stage reaction mixture (based upon the determination of optimal conditions described below) contained 0·1 M-tris (hydroxymethylaminomethane), pH 8·6; 14 mM-$MgCl_2$; 15 mM-UTP; 20 mM-glucose-1-P; 10 mM-KF; 0·01 % bovine serum albumin, and 0·1–0·6 μg rabbit brain (dry weight) per μl (final concentrations). After incubating for 1 hr at 38°, the reaction mixture was heated for 2 min in boiling water, and portions were transferred to 20 vols of the UDP-glucose dehydrogenase reagent for estimation of the UDP-glucose formed.

The UDP-glucose dehydrogenase system contained 0·1 M-tris, pH 8·8; 1 mM-EDTA; 0·3 mM-DPN^+; and 0·01 % bovine serum albumin (final concentrations). UDP-glucose dehydrogenase was added to a concentration of 210 units per ml so that the initial rate of UDP-glucose oxidation was 0·5 m-mole per l. per hr. With 0·06 mM-UDP-glucose the reaction was more than 99 per cent complete in 30 min at 38° since the Michaelis constant is 0·02 mM.

Typically in a micro-assay, the first-stage reaction mixture was 5 μl; a 1·2 μl portion (containing less than $1{\cdot}5 \times 10^{-9}$ mole UDP-glucose) was added to 24 μl of the second-stage reagent. The DPNH formed was estimated by the fluorometric method of LOWRY, ROBERTS, and KAPPHAHN (1957) as follows: the sample was heated for 15 min at 60° after adding 50 μl of 0·6 M-phosphate buffer (1 : 2 molar ratio Na_3PO_4 : K_2HPO_4) to destroy excess DPN^+. A 50 μl portion was removed to 200 μl of 7·5 N-NaOH containing 0·01 % H_2O_2 in fluorometer tubes and heated for 15 min at 60° to develop the fluorescent product. Water was added to 1·0 ml, and the tubes were read in the Farrand fluorometer. Appropriate blanks and standards of UDP-glucose and DPNH were included. For larger samples, containing 10 to 25×10^{-9} mole UDP-glucose in 0·5 ml of the UDP-glucose dehydrogenase reagent, the DPNH formed was measured spectrophotometrically.

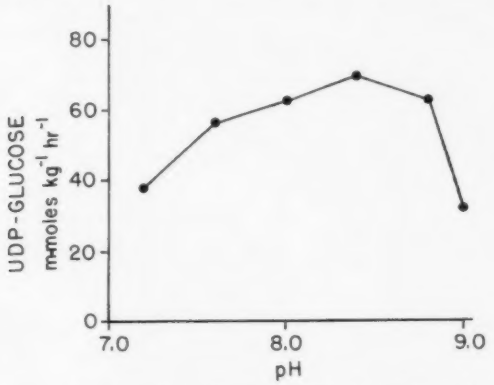

FIG. 1.—pH optimum of UDP-glucose formation in brain. The incubation mixture contained (final concentrations) 70 mM-tris; 5 mM-$MgCl_2$; 11 mM-KF; 4 mM-UTP; 3 mM-glucose-1-P; and 1 mg rabbit brain in a total volume of 90 μl. After 1 hr at 38° the mixture was heated for 2 min in boiling water, centrifuged, and 25 μl of the supernatant fraction were transferred to 0·5 ml of UDP-glucose dehydrogenase reagent. Values given are the means of triplicates

RESULTS

The dependence of UDP-glucose formation upon pH is shown in Fig. 1. In the optimal range of pH between 8·2 and 8·8, tris and glycine buffers gave identical activities. Potassium fluoride, added to inhibit destruction of UTP and glucose-1-P by phosphatase, enhanced the activity by 25 per cent. Bovine serum albumin was added to stabilize enzymes at the low concentrations employed.

From measurements of the rate of formation of UDP-glucose with varied concentrations of UTP and glucose-1-P (Fig. 2), Michaelis constants (K_m) of 2·5 mM-UTP and 0·6 mM-glucose-1-P were calculated.

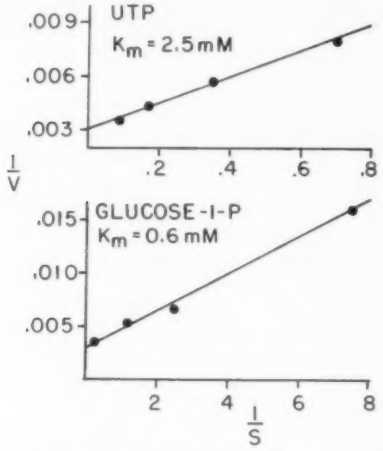

FIG. 2.—Dependence of UDP-glucose formation upon concentrations of UTP and glucose-1-P. The substrate concentration, *S*, is expressed as mM and the velocity, *V*, as m-moles UDP-glucose/kg/hr. The incubation mixture contained final concentrations of 100 mM-tris, pH 8·6; 10·6 mM-$MgCl_2$; 8 mM-KF; 0·01% bovine serum albumin, and 20 μg rabbit brain in 15 μl total volume. The concentration of glucose-1-P was 4·2 mM in the experiment with varied UTP concentrations, and the concentration of UTP was 4·1 mM in the experiment with varied glucose-1-P concentrations. After 1 hr at 38° the mixture was heated for 2 min in boiling water, and portions were transferred to the UDP-glucose dehydrogenase reagent. Values given are the means of triplicates.

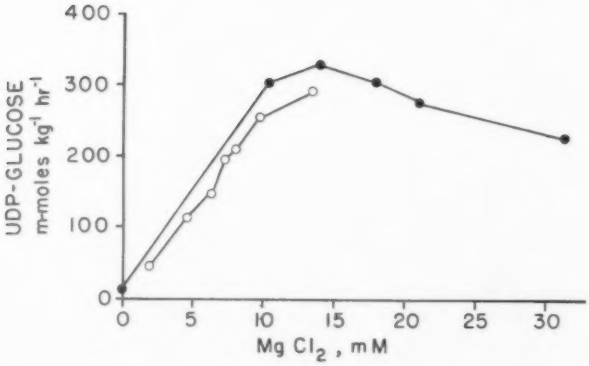

FIG. 3.—The effect of $MgCl_2$ concentration upon UDP-glucose formation. The incubation mixture contained final concentrations of 0·1 M-tris, pH 8·6; 22 mM-glucose-1-P; 17 mM-UTP; 0·01% bovine serum albumin, and 15 μg rabbit brain in a total volume of 15 μl. After 1 hr at 38° the mixture was heated for 2 min in boiling water, and portions were transferred to the UDP-glucose dehydrogenase reagent. The values for each of 2 experiments are the means of triplicates.

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The optimal $MgCl_2$ concentration was 14 mM (Fig. 3). This unusually high apparent Mg^{++} requirement is a function of complex formation with UTP. Fig. 4 illustrates the decrease in rate of UDP-glucose formation with increasing UTP concentrations when the ratio of UTP to $MgCl_2$ exceeds a value of approximately 1·0. In similar experiments with glucose-1-P in concentrations up to 20 mM, there was no decrease in the formation rate. The formation of UDP-glucose was proportional to time for at least 4 hr. Approximately one-third of the activity was lost from brain homogenates in water stored at −20° for 3 weeks. There was no loss of activity on lyophilization.

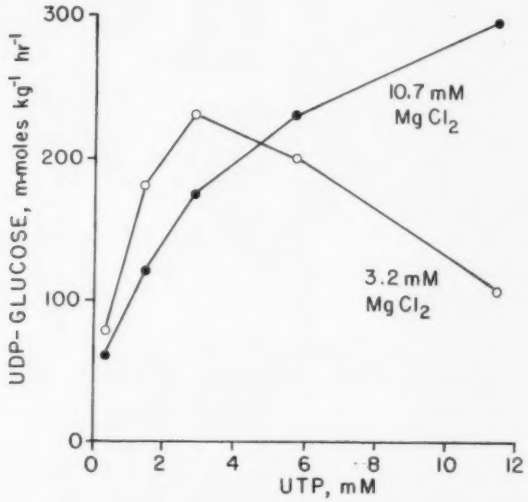

FIG. 4.—The dependence of UDP-glucose formation upon UTP and $MgCl_2$ concentrations. In addition to the indicated concentrations of UTP and $MgCl_2$, the incubation mixture contained 100 mM-tris, pH 8·6; 4·2 mM-glucose-1-P; 8·1 mM-KF; 0·01% bovine serum albumin, and 20 μg rabbit brain in a total volume of 15 μl. After 1 hr at 38° the mixture was heated for 2 min in boiling water and portions were transferred to the UDP-glucose dehydrogenase reagent. The values given are the means of 6 determinations.

The optimal range of brain dilution is between 300- and 600-fold in the incubation mixture (Table 1). At a brain dilution of 600-fold the UDP-glucose concentration in the UDP-glucose dehydrogenase reagent was approximately 0·03 mM, which gave an increment in fluorometer reading of twice the blank.

The rate of formation of UDP-glucose for each of two rabbit brain homogenates was 310 m-moles per kg per hr. Additional data obtained with this method for different areas of the rabbit central nervous system are presented in the following paper (BRECKENRIDGE and CRAWFORD, 1961) in conjunction with data obtained for other enzymes of glycogen metabolism.

The incubation mixture contained 100 mM-tris, pH 8·6; 15 mM-UTP; 20 mM-glucose-1-P; 14 mM-$MgCl_2$; 10 mM-KF; 0·01% bovine serum albumin, and the indicated dilutions of brain in a total volume of 15 μl. After 1 hr at 38° the mixture was heated for 2 min in boiling water, and portions were transferred to the UDP-glucose

TABLE 1.—RANGE AND REPRODUCIBILITY OF UDP-GLUCOSE PYROPHOSPHORYLASE ASSAY IN BRAIN

Brain dilution	UDP-glucose formed (m-moles/kg/hr)
150	213
300	252
600	254
	258
	243
	252
	236
	243
	248 ± 3
1200	222
2400	211

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dehydrogenase reagent. At a brain dilution of 600-fold (wet wt/vol.) the individual determinations are given, together with the mean and standard error of the mean. The remaining values are means of triplicates.

DISCUSSION

The assay described for UDP-glucose pyrophosphorylase measures the reaction in the direction which probably prevails in tissue, *i.e.*, the formation of UDP-glucose. For estimates of UDP-glucose pyrophosphorylase activity in crude systems this may afford an advantage in comparison with the reverse reaction. Tissue homogenates liberate glucose-1-P from UDP-glucose in the absence of added pyrophosphate (GINSBURG, 1958). In the present work with brain the formation of UDP-glucose was dependent upon the addition of both UTP and glucose-1-P. In addition some of the complex interactions of Mg^{++}, pyrophosphate, and pH encountered in measuring the pyrophosphorolysis of UDP-glucose may be avoided.

While the assay has been especially useful for studies on the quantitative histochemistry of the brain, it may be equally useful in other investigations involving this enzyme e.g. equilibrium measurements (TURNER and TURNER, 1958), and the question of the relative velocities of the forward and reverse reactions with different preparations (SMITH and MILLS, 1954; REID, 1959). The second stage of the assay can also be used for micro-determination of UDP-glucose in other situations.

The observed level of activity for UDP-glucose formation in rabbit brain, about 300 m-moles per kg per hr, is more than 3 times the rate of the reverse reaction in rat brain (VILLAR-PALASI and LARNER, 1960*b*), although the latter measurements were made at lower temperature and pH than those employed here. The observed level of activity is also more than 10 times that of the enzyme synthesizing glycogen from UDP-glucose in rabbit brain homogenate (BRECKENRIDGE and CRAWFORD, 1960).

SUMMARY

1. A micro-assay for uridine diphosphate glucose pyrophosphorylase is described in which uridine diphosphate glucose formation from uridine triphosphate and

glucose-1-phosphate is estimated by the fluorometric measurement of DPNH formed on oxidation of uridine diphospposphate glucose with diphosphopyridine nucleotide (DPN^+) in the presence of the dehydrogenase.

2. Approximately 300 m-moles UDP-glucose per kg per hr are formed by rabbit brain. The optimal pH is between 8·2 and 8·8, and the optimal $MgCl_2$ concentration is 14 mM. Half-maximal activity is obtained with 0·6 mM-glucose-1-P or 2·5 mM-uridine triphosphate.

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THE QUANTITATIVE HISTOCHEMISTRY OF THE BRAIN

ENZYMES OF GLYCOGEN METABOLISM*

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THE concept of glycogen metabolism which developed after the discovery of glycogen synthesis from uridine diphosphate glucose by LELOIR and CARDINI (1957) is shown diagrammatically (Fig. 1). The uridine-linked synthesis of glycogen, first shown to occur in liver, has also been found in brain and other tissues (BRECKENRIDGE and CRAWFORD, 1959, 1960; LELOIR, OLAVARRÍA, GOLDEMBERG and CARMINATTI, 1959).

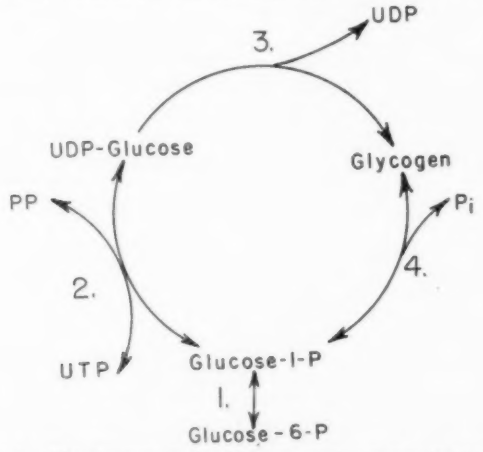

FIG. 1.—The glycogen cycle. Enzymes active at the sites indicated are: 1. phosphoglucomutase, 2. UDP-glucose pyrophosphorylase, 3. glycogen synthetase, 4. phosphorylase. Abbreviations used are: glucose-6-phosphate, glucose-6-P; glucose-1-phosphate, glucose-1-P; uridine triphosphate, UTP; uridine diphosphate, UDP; uridine diphosphate glucose, UDP-glucose; ortho phosphate, P_i; pyrophosphate, PP.

The cyclic sequence of reactions in which glycogen is synthesized from UDP-glucose and degraded by phosphorylase was noted by VILLAR-PALASI and LARNER (1958) and by LELOIR *et al.* (1959). Additional evidence for the general applicability of the glycogen cycle in mammalian tissues was reviewed recently by STETTEN and STETTEN (1960). This schematic description applies to the α-1,4′ linkages and does not include the α-1,6′ branching linkages.

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Differences in glycogen metabolism between different regions of the brain have been suggested by a variety of earlier studies (FERRIS and HIMWICH, 1946; SHIMIZU and KUBO, 1957; SVORAD, 1959).

The present paper describes quantitative measurements of the activities of the glycogen cycle enzymes and of phosphoglucomutase in several regions of the rabbit central nervous system. Advantages and limitations of the quantitative histochemical procedures, in comparison with other methods of studying histologically complex tissues, have been discussed by ROBINS and SMITH (1953) and are considered further in the discussion for the particular case of glycogen metabolism.

METHODS

The preparation of tissue and procedures for the enzymic analysis of histologically defined areas of brain have been described by LOWRY *et al.* (1953, 1954*a*, 1954*b*).

After decapitation, portions of 2 adult white rabbit brains were removed and frozen in dichlorodifluoromethane (Freon-12) chilled in liquid nitrogen. Sections were cut at 20 μ and dried at $-40°$; pieces were dissected from the following regions: mid-cervical spinal cord dorsal columns (white) and ventral horn (grey), molecular and granular layers of the cerebellar cortex, stratum griesum superficiale of the superior colliculus, caudate nucleus, temporal cerebral cortex (including all layers), and the optic nerve. Some variation in glycogen metabolism might be anticipated in these areas, which included regions with predominantly cell bodies (granular layer of cerebellum), dendrites (molecular layer of cerebellum), and myelinated axons (optic nerve and dorsal columns).

A summary is given of the incubation conditions for the 4 enzymes measured (Table 1). Procedures for phosphoglucomutase, UDP*-glucose pyrophosphorylase, and phosphorylase were used exactly as described in the references cited (Table 1). The procedure for glycogen synthetase is based upon the optimal conditions, validated for use on a micro scale, previously described (BRECKENRIDGE and CRAWFORD, 1960). Brain sections weighing 0·5—5·0 μg were incubated for 1 hr at 38° in 1·2 μl of the reagent, covered with hexane. The reaction was stopped, after removal of the hexane and the addition of 5 μl of water, by heating for 2 min in boiling water. The UDP formed was estimated by means of the reactions catalysed by pyruvic phosphokinase and lactic dehydrogenase:

$$\text{phosphoenolpyruvate} + \text{UDP} \rightarrow \text{pyruvate} + \text{UTP}$$

$$\text{pyruvate} + \text{DPNH} \rightarrow \text{lactate} + \text{DPN}^+$$

The combined reagent for these reactions contained 30 mM-glycylglycine, pH 7·4; 70 mM-KCl; 15 mM-$MgCl_2$; 0·3 mM-DPNH; 0·3 mM-phosphoenolpyruvic acid, and sufficient lactic dehydrogenase containing pyruvic phosphokinase to convert more than 99% of 0·05 mM (final concentration) UDP to UTP in 10 min at 38°. Five μl of this reagent were added to each sample, and after 10 min at 38°, 5 μl of N-HCl were added to destroy DPNH. The DPN^+ was measured fluorometrically in a final volume of 1·0 ml, (LOWRY, ROBERTS, and KAPPHAHN, 1957). Appropriate blanks and standards of UDP and DPN^+ were included. At the UDP-glucose concentration employed, measured velocity was 73% of that with excess substrate. Higher concentrations of UDP-glucose increased the blank proportionally more than velocity, owing to a small contamination of the UDP-glucose with UDP.

Lipid-free dry weight was determined gravimetrically after treatment with absolute alcohol and hexane (LOWRY, 1953).

RESULTS

Of the four enzymes measured, phosphoglucomutase shows the highest level of activity and glycogen synthetase the lowest (Table 2). In comparison with glycogen synthetase, the level of phosphoglucomutase in brain homogenate is 57 times as great, phosphorylase is 31 times as great, and UDP-glucose pyrophosphorylase is 12 times as great. There is close agreement in the values for each brain area for two different rabbits.

* *Abbreviations used*: UDP, uridine diphosphate; UTP, uridine triphosphate; DPN, diphosphopyridine nucleotide.

TABLE 1.—INCUBATION CONDITIONS FOR ENZYMES OF GLYCOGEN METABOLISM

Enzyme	pH	Buffer	Substrate	Other additions	Principle of measurement	Reference
Phospho-glucomutase	7·6	10 mM-tris (hydroxy-methyl) amino-methane	2 mM-glucose-1-P	2·2 mM-$MgCl_2$; 2·4 mM-dimercaprol; 0·04 mM-glucose-1:6-diphosphate; 0·05% bovine serum albumin	Glucose-6-P reacted with glucose-6-P-dehydrogenase and TPN^+. Fluorometric measurement of TPNH formed.	LOWRY *et al.* (1961)
UDP-glucose pyrophospho-rylase	8·6	100 mM-tris (hydroxy-methyl) amino-methane.	15 mM-UTP, 20 mM-glucose-1-P	10 mM-KF; 0·01% bovine serum albumin, 14 mM-$MgCl_2$	UDP-glucose reacted with UDP-glucose dehydrogenase and DPN^+. Fluorometric measure-ment of DPNH formed.	BRECKENRIDGE, SCOTT, STROMINGER, and CRAWFORD. (1961)
Glycogen synthetase	7·9	100 mM-glycyl-glycine.	7·3 mM-UDP-glucose	13 mM-glycogen (as glucose); 9 mM-glucose-6-P; 15 mM-cysteine; 6 mM-ethylenediamine-tetra-acetate.	UDP measured with pyruvic phosphokinase-lactic dehydrogenase system with fluorometric measurement of DPN^+.	BRECKENRIDGE and CRAWFORD (1960)
Phosphory-lase	6·7	substrate	30 mM-glucose-1-P	28 mM-glycogen (as glu-cose); 1 mM-5′-adenlyic acid, 50 mM-NaF; 15 mM-cysteine	Orthophosphate measured spectrophotometrically with molybdic acid reagent.	BUELL *et al.* (1958)

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TABLE 2.—DISTRIBUTION OF ENZYMES OF GLYCOGEN METABOLISM IN RABBIT CENTRAL NERVOUS SYSTEM

	Lipid	Phospho-glucomutase	UDP-glucose pyrophospho-rylase	Glycogen synthetase	Phosphory-lase
Dorsal column	3·52 ± 0·07	19·2 ± 1·1	1·97 ± 0·09	0·402 ± 0·013	6·3 ± 0·2
	3·59 ± 0·09	19·0 ± 0·3	1·52 ± 0·04	0·394 ± 0·017	6·9 ± 0·6
Optic nerve	1·61 ± 0·03	12·3 ± 0·3	1·24 ± 0·02	0·185 ± 0·008	4·7 ± 0·1
	1·79 ± 0·02	11·2 ± 0·2	0·91 ± 0·05	0·229 ± 0·018	4·5 ± 0·2
Ventral horn	1·25 ± 0·03	15·9 ± 0·4	2·34 ± 0·07	0·342 ± 0·012	10·8 ± 0·2
	1·19 ± 0·02	15·6 ± 0·3	2·14 ± 0·04	0·428 ± 0·024	9·3 ± 0·5
Superior colliculus	0·77 ± 0·06	14·5 ± 0·5	2·03 ± 0·05	0·315 ± 0·024	10·2 ± 0·3
	0·73 ± 0·04	14·8 ± 0·2	1·49 ± 0·07	0·296 ± 0·016	8·7 ± 0·7
Caudate nucleus	0·72 ± 0·05	12·9 ± 0·2	2·07 ± 0·05	0·281 ± 0·023	9·6 ± 0·3
	0·77 ± 0·02	11·7 ± 0·3	1·91 ± 0·13	0·287 ± 0·009	7·7 ± 0·5
Molecular layer, cerebellum	0·72 ± 0·01	15·4 ± 0·3	5·16 ± 0·14	0·438 ± 0·020	15·8 ± 0·4
	0·70 ± 0·01	15·6 ± 0·1	4·34 ± 0·14	0·422 ± 0·009	12·8 ± 0·6
Cerebral cortex	0·58 ± 0·01	13·2 ± 0·2	2·41 ± 0·07	0·242 ± 0·012	7·0 ± 0·3
	0·60 ± 0·01	13·2 ± 0·2	1·65 ± 0·09	0·250 ± 0·015	7·1 ± 0·2
Granular layer, cerebellum	0·46 ± 0·02	12·4 ± 0·2	1·87 ± 0·13	0·245 ± 0·014	7·8 ± 0·1
	0·51 ± 0·02	11·8 ± 0·3	1·80 ± 0·15	0·234 ± 0·026	7·0 ± 0·3
Brain homogenate	1·0	14·7	3·05	0·258	7·9

Enzyme values are given as moles of substrate converted per kg lipid-free dry wt per hr. Lipid values are given as kg lipid per kg lipid-free dry weight. In most cases the mean of 6 determinations is given, followed by the S.E.M. The 2 recorded sets of values were obtained from 2 different rabbits. In each instance the first figure is referable to one rabbit, the second to the other. The lipid value for average brain is from an earlier study.

Levels of phosphorylase tend to correspond with those of UDP-glucose pyrophosphorylase, and levels of phosphoglucomutase tend to correspond with those of glycogen synthetase (Fig. 2). With the exception of the dorsal columns, areas which are relatively high in glycogen synthetase are high in phosphorylase as well. The molecular layer of cerebellum is unusual in exhibiting a level of UDP-glucose pyrophosphorylase more than twice that of any of the other areas examined.

There is no consistent variation in the measured enzyme levels with lipid content or along the neuraxis.

DISCUSSION

It is clear that the enzymes of the glycogen cycle are present with sufficient activity in the central nervous system to account for maximal rates of glycogen accumulation and disappearance in intact brain. A similar conclusion was reached by VILLAR-PALASI and LARNER (1960) on the basis of lower values obtained for rat brain homogenate with different assay procedures.

Maximal rates of glycogen accumulation in cerebral cortex appear not to exceed 2 m-moles hexose per kg/hr *in vitro* (MCILWAIN and TRESIZE, 1956) or *in vivo*

(KŘIVÁNEK, 1958). For comparison, the enzyme activities reported here may be converted to an approximate fresh weight basis by dividing by 10 since the lipid-free dry weight for various regions of brain is a fairly constant fraction (10–12%) of the wet weight. The value for glycogen synthetase in cerebral cortex on a wet weight basis is 25 m-moles per kg/hr. Within the limitation of comparison between species and preparations, enzyme activity is 12 times as great as that needed for the maximal rate of accumulation of glycogen in the tissue.

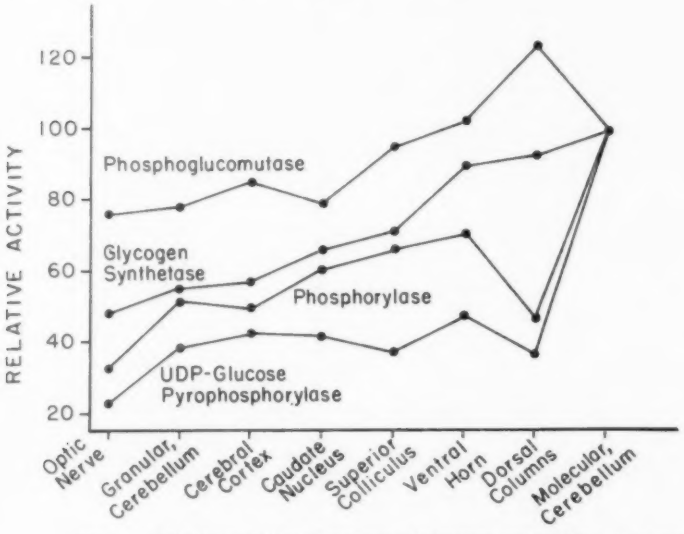

FIG. 2.—Relative activities of enzymes of glycogen metabolism. For each enzyme the average value on a lipid-free dry weight basis for the molecular layer of the cerebellum is arbitrarily designated as 100. The connecting lines are intended to facilitate comparisons and do not imply continuity between the points.

The relative levels of enzyme activities (Fig. 2) may be useful indices in further studies, though no firm interpretation may be derived from them at present. For example, does the high UDP-glucose pyrophosphorylase level in the molecular layer of cerebellum indicate an increased requirement for UDP-glucose to form UDP-glucuronate or UDP-galactose as intermediates in glucuronide or cerebroside synthesis?

It seems likely that glycogen synthetase is the step controlling the rate of glycogen formation. It has the lowest activity of the enzymes concerned when all are compared under optimal conditions of substrate and pH. Glycogen synthetase of brain, like that of other tisues, is activated by glucose-6-P. Thus the indirect regulator of glycogen synthesis could be the tissue concentration of glucose-6-P.

Glycogen disappears from brain at a rate of at least 100 m-moles hexose per kg per hr (KERR and GHANTUS, 1937), and possibly at a much higher rate (MCILWAIN, 1955). This would suggest that phosphorylase may be utilized near maximal capacity, for its level is 700 m-moles per kg/hr in cerebral cortex on a wet weight basis.

It is likely that a phosphorylase-activating system controls the rate of glycogenolysis in brain. It has been shown by SUTHERLAND and RALL (1958) that the cyclic

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adenine ribonucleotide activator for phosphorylase kinaseis enzymically synthesized and degraded in brain. CORI and CORI (1940) obtained purified brain phosphorylase in the form requiring adenylic acid for activity. Since the phosphorylase activity of brain homogenate without added adenylic acid is more than 50% of that with the complete phosphorylase reagent, there is presumably also an active form of brain phosphorylase which does not require adenylic acid. Thus the evidence, while incomplete, does suggest that the control of glycogenolysis in brain is by means of a system similar to that in liver and muscle.

The phosphorylase levels of optic nerve and dorsal columns, as well as those of other myelinated tracts studied by BUELL *et al.* (1958) and by ROBINS, SMITH, and JEN (1957), are generally lower than those of grey areas. Thus the concept that myelinated tracts, in comparison with grey matter, may use glycogen for a special role (ROBINS, 1960) depends upon the relative deficiency of hexokinase for direct glucose utilization, and gains no support from the phosphorylase levels alone.

The study of glycogen metabolism in brain requires special appraisal of methods because of the high velocity of change of substrate concentrations and because of the intermingling of histological structures. In the amount of time taken in the present study for dissecting and freezing, brain enzyme levels are generally unchanged (SMITH, ROBINS, EYDT and DAESCH, 1957). Thus, quantitative data of specific reactions may be obtained for defined areas. In contrast, the concentration of glycogen may change at a rate of 18% of the initial content per sec (MCILWAIN, 1955), and is affected by a large number of variables. Consequently rapid *in situ* freezing is required. The measured concentration of glycogen may reflect the ratio of synthesis and degradation, but it gives no indication of either rate. Finally, technically adequate studies of brain glycogen to date have been limited to brain samples weighing several mg, so that, for example, any differences associated with the distinct enzyme patterns of the molecular and granular layers of the cerebellum, observed in these studies, were obscured. Investigations of brain by the staining of polysaccharides have revealed interesting localized deposits, but the above considerations have been invariably neglected so that at best a non-specific, non-quantitative, index of brain glycogen metabolism has been obtained.

SUMMARY

1. Quantitative measurements of four enzymes of glycogen metabolism, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, glycogen synthetase, and phosphorylase, for eight different regions of the rabbit central nervous system are described.

2. The levels of enzymic activities occur in distinctive patterns with magnitudes sufficient to account for maximal rates of glycogen accumulation and disappearance *in vivo*.

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THE CONTENT OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN BRAIN OF NORMAL AND ANAESTHETIZED RATS: A CRITICAL STUDY OF SOME FACTORS INFLUENCING THEIR ASSAY

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SPECIAL procedures are required in the analysis of labile phosphate compounds of brain because of the rapid breakdown of these substances during the manipulation of the animal prior to killing and before the enzymes of the brain are denatured by a protein precipitant (MCILWAIN, 1952). KERR (1935) observed that CP* levels in dog and cat brain were extremely low unless the animal was anæsthetized, the skull removed under artificial respiration, and liquid oxygen poured on the brain surface. With smaller animals, immersion of the whole animal in liquid air was required to obtain high levels of CP. LEPAGE (1946*a*, *b*), using the immersion procedure, studied levels of labile phosphate compounds and carbohydrate intermediates in rat tissues, and found that animals which were not anæsthetized with pentobarbitone before immersion possessed considerably lower brain levels of ATP and CP, and higher levels of ADP and AMP than those which had been anæsthetized. MCILWAIN, BUCHEL, and CHESHIRE (1951) showed that if guinea pigs were decapitated and the brain was dropped into liquid oxygen within twenty seconds, there was almost complete disappearance of CP from the tissue. KRATZING and NARAYANASWAMI (1953), comparing whole body immersion with guillotining followed by immersion of the head, reported a slight depletion of brain CP and ATP in guinea pigs. The number of animals employed was too small to permit evaluation of the significance of these differences. KORANSKY (1958), using the decapitation procedure, found considerably lower levels of labile phosphate compounds in rat brain than those reported by workers who killed animals by whole body immersion in liquid nitrogen.

In the present report, an evaluation of some of the factors involved in the preservation and analysis of labile phosphate compounds of brain is attempted with the use of specific enzymic methods for the analysis of adenine nucleotides. The levels of labile phosphate compounds in ether- and pentobarbitone-anæsthetized rats and studies on the enzymes involved in their catabolism are presented.

METHODS

Male, white, Sprague-Dawley rats, 42–100 g in weight, were used in these experiments. In the immersion procedure, the animal was held gently 30–60 sec to maintain a brief state of outward calm. The rat was then suddenly plunged head first into liquid oxygen in a Dewar flask. Complete freezing, as shown by the cessation of active gas evolution, occurred in 1–2 min, whereupon the frozen carcass was removed, and the head cut off by means of a cold steel chisel. The brain was rapidly chiselled free from the skull. During this procedure the skull and brain particles were kept submerged in liquid

* Abbreviations used are: CP, creatine phosphate; OD, optical density; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

oxygen except for the brief intervals during which the dissection was performed. The brain was pulverized several times in a cold steel homogenizer and cooled in liquid oxygen, and all or a portion of the frozen powder was poured into a tared centrifuge tube containing 5·0 ml of cold 0·4 N-perchloric acid. The tube was shaken vigorously to break the ice crust formed at the interface of tissue and acid and to expose the tissue to the protein precipitant as rapidly as possible. The breaking of the ice interface occasionally required several seconds, the length of time being in general directly related to the size of the tissue sample. The tared centrifuge tube was reweighed, and the weight of the tissue sample obtained by difference. In some experiments, a tared glass homogenizer containing 5·0 ml of 0·4 N-$HClO_4$ was used in place of the centrifuge tube, and the frozen, powered tissue was briefly homogenized before weighing the homogenizer plus tissue. The tissue weight was obtained by difference. All of the above procedures were carried out in the cold room at approximately 4°. After being in contact with the acid for about 30 min at 2°, during which time the tissue was carefully crushed with a flattened stirring rod, the sample was centrifuged for 15 min at 900 **g** in a refrigerated centrifuge at 2°. The supernatant fluid was decanted off, and the precipitate was carefully crushed twice and mixed with a stirring rod in 2 ml of 0·2 N-$HClO_4$ each time, each wash being spun at 900 **g** for 10 min. The supernatant fluid and washes were combined and neutralized to approximately pH 7 with KOH with the spot-plate technique and bromthymol blue as indicator. The neutralized material was stored in the cold for 30 min and spun in the refrigerated centrifuge at 900 **g** for 15 min to separate the $KClO_4$ precipitate. The volume of the supernatant fluid was measured and subsequently analysed for adenine nucleotides and CP. Another group of animals was killed by decapitation with a large pair of scissors. The animal was held in such a way that the severed head fell directly into liquid oxygen. The interval between the initiation of decapitation and the submersion of the head in liquid oxygen was approximately 1 sec or less. Animals were anæsthetized by placing them in a large jar and pouring ether on a wick suspended from the jar cover. The animals were in deep anæsthesia, characterized by complete lack of responsiveness to painful stimuli, for at least 10 min, after which time they were dropped into liquid oxygen. A final group of animals was anæsthetized by the subcutaneous administration of sodium pentobarbitone, 7–8 mg/100 g body weight. The animals were unresponsive to painful stimuli within 10–15 min and remained so for 15–30 min, after which time they were dropped into liquid oxygen. None of the anæsthetized animals showed any signs of severe respiratory depression or oxygen deficiency at the time of immersion in liquid oxygen. Subsequent to the immersion in liquid oxygen the brain tissue of all groups was handled in exactly the same manner as that of the controls.

Assay of labile phosphate compounds. AMP, ADP and ATP were assayed by modifications of the methods of KALCKAR (1947) and ALBAUM and LIPSHITZ (1950). Adenylic acid deaminase was prepared according to the method of SCHMIDT (1928). Myokinase was made according to the method of KALCKAR (1947). Hexokinase was obtained from a commercial source. Each enzyme preparation was demonstrated to be free of interfering enzymes. Enzymes were diluted to a concentration which enabled the reaction to go to completion in less than 40 min, 0·02 ml of each enzyme being used. The following reagents were pipetted into a quartz cuvette: 0·1 ml 0·1 M-$MgCl_2$, 0·2 ml 1·2 M-KCl, 1·0 ml 0·1 M-malonate buffer, pH 5·9; 0·4 ml 0·5 M-glucose, an appropriate volume of sample; and water to bring the total volume to 3·7 ml. The initial OD readings at 265 mμ and 251 mμ were recorded, and 0·02 ml adenylic acid deaminase was added. The resulting OD change at 265 mμ was noted; it reflected the amount of AMP in the sample. Myokinase, 0·02 ml, was added and the OD change at 265 mμ was determined; it was proportional to one-half the amount of ADP in the sample. Finally, 0·02 ml hexokinase was added; the OD change obtained was proportional to one-half the ADP and all the ATP in the aliquot. Blanks and known mixtures of nucleotides were run with each series, and corrections were made for the slight non-specific OD changes at 251 mμ, due to the addition of enzymes. Readings were made in a Beckman DU spectrophotometer. Free and total creatine were determined by the method of ENNOR and ROSENBERG (1952), the difference between these representing the amount of CP in the sample. All results are expressed in μmole/g of brain tissue.

Enzyme studies. AMP, ADP, ATP and CP, the substrates in these investigations, were obtained from commercial sources. The nucleotides were tested for purity by the enzymic methods described, and by ion exchange chromatography (MOMMAERTS, 1958). CP was similarly checked by the procedure of ENNOR and ROSENBERG (1952). Those preparations not found to be sufficiently pure were either rejected or further purified. Substrates were dissolved in water, neutralized to pH 7·4, and

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diluted to a final concentration of 10 mM. Whole rat brains were homogenized in 0·2 M-Tris buffer, pH 7·6, containing 0·01 M-$MgCl_2$, a Potter-Elvehjem glass homogenizer being used. Either 10% or 4% homogenates were used. The homogenates, 0·5 to 1·0 ml, were placed in flasks with 4·0 ml of the buffer mixture and sufficient water to make the final total volume 5·5 ml. The flasks were thermoequilibrated in a Dubnoff metabolic shaking incubator for 5 min, and 1·0 ml of either one or two of the substrates was added. Samples were removed at 0, 3, 6, and 10 min, pipetted into 0·4 N-$HClO_4$ and centrifuged at 2° for 10 min. The precipitate was washed once with 0·2 N-$HClO_4$, centrifuged,

TABLE 1.—BRAIN LABILE PHOSPHATE COMPOUNDS IN CONTROL, ETHER- AND PENTOBARBITONE-TREATED RATS. IMMERSION METHOD

Group	AMP	ADP	ATP	CP	Free creatine	Mean brain sample size (g)
	(μmole/g)					
Control (15)*	0·42	0·66	1·22	2·02	6·69	0·70
S.D.†	0·05	0·04	0·11	0·14	0·17	
Pentobarbitone (16)	0·45	0·64	1·34	2·56‡	6·75	0·73
S.D.	0·05	0·07	0·15	0·14	0·21	
Ether (16)	0·45	0·57	1·23	2·47§	6·65	0·73
S.D.	0·05	0·04	0·08	0·15	0·15	

* Number of animals in each group in parentheses.
† S.D. = Standard deviation of the mean.
‡ $P < 0·02$ that difference between pentobarbitone-treated group and control group is due to chance.
§ $P < 0·05$ that difference between ether-treated group and control group is due to chance.

and the supernatant fluids were combined and neutralized with KOH. The $KClO_4$ precipitate formed was removed by centrifugation. Inorganic phosphorus was determined by the method of FISKE and SUBBAROW (1925) unless CP was used as substrate. In the latter circumstance inorganic phosphorus was determined by the modified method of BERENBLUM and CHAIN (1938) (WEIL-MALHERBE and GREEN, 1951), and free creatine by the method of ENNOR and ROSENBERG (1952). Enzyme activity was determined by plotting product formed against time, and drawing the line which best fitted the plotted values. Results were calculated from this line and are expressed in μmoles product formed per g tissue per sec.

Analysis of the variability in concentrations of labile phosphate compounds. The method of regression analysis was employed to evaluate possible factors contributing to the variability in the concentrations of AMP, ADP, ATP and CP among brain samples. Apparent correlations were evaluated by the method of least squares, the equations of the regression lines were calculated, and the significance of the correlations was determined (Figs. 1–3) (SNEDECOR, 1956). Among the factors evaluated were animal size, brain size, brain sample size, and level of excitability prior to immersion in liquid oxygen.

RESULTS

Control animals. Immersion procedure. ATP is the predominant adenine nucleotide in rat brain, but there are considerable amounts of AMP and ADP (Table 1). The CP level in rat brain averages about 2 μmole/g, while total brain creatine averages about 9 μmole/g. The considerable variation in the relative amounts of the various nucleotides and CP in the brains of different control rats is reflected in the large standard deviations. An analysis of the data reveals that the large variability is neither related to the animal size nor to the level of excitability before immersion, but seems to be best correlated with the brain sample size. To evaluate this further, a number of animals were killed in the manner described, the whole brain was pulverized, and a fraction of this was treated with perchloric acid and analysed in the usual manner

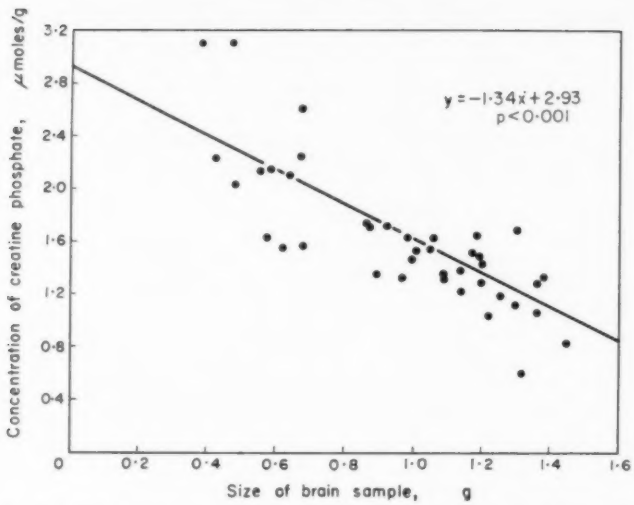

FIG. 1.—Relation between size of brain sample and concentration of creatine phosphate in brain. Points are individual values experimentally obtained. The line and the equation of the line were obtained by regression analysis. *P* is the probability that the slope of the regression line is not different from zero.

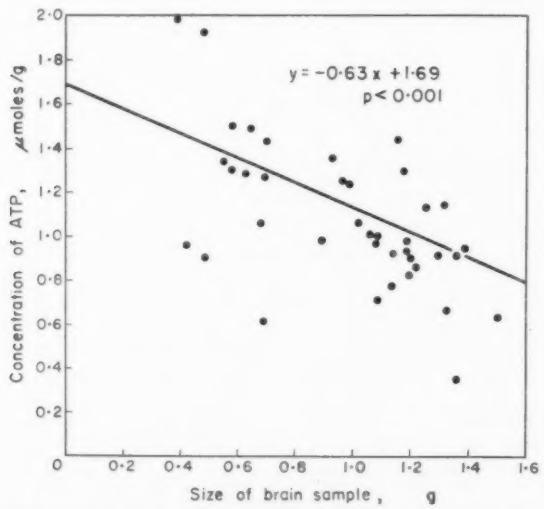

FIG. 2.—Relation between size of brain sample and concentration of adenosine triphosphate in brain.

(Figs. 1–3). Levels of both CP and ATP are inversely related to the size of brain sample (Figs. 1 and 2), whereas the level of AMP is directly related to the size of the brain sample (Fig. 3). The levels of ADP and free creatine bear no significant relationship to the size of the sample.

The critical effect of the ratio between size of brain sample and volume of extracting fluid was demonstrated by varying each. Larger amounts of brain sample can be used for analysis if the volumes of extracting fluid are proportionately increased. The

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levels of phosphorylated compounds obtained are fairly constant for a definite ratio of brain sample size to volume of perchloric acid used for extraction. Optimal extractions are obtained where the ratios of perchloric acid to brain sample size in the three extractions are 10:1, 5:1 and 5:1.

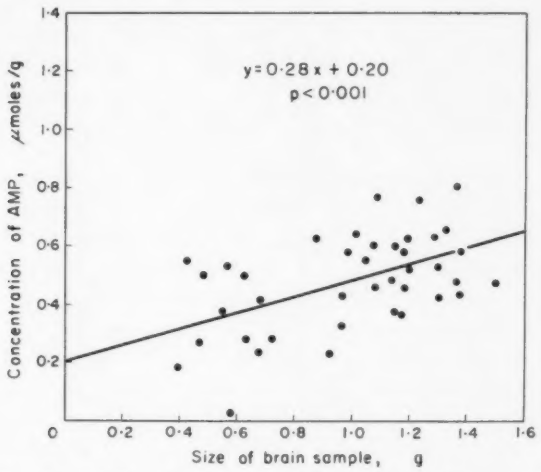

FIG. 3.—Relation between size of brain sample and concentration of adenosine monophosphate in brain.

TABLE 2.—BRAIN LABILE PHOSPHATE COMPOUNDS IN CONTROL RATS. DECAPITATION METHOD

	AMP	ADP	ATP	CP	Free creatine	Mean brain sample size (g)
	(μmole/g)					
Mean*	1·00‡	0·86§	0·58‡	0·67‡	8·85‡	0·70
S.D.†	0·05	0·06	0·04	0·06	0·25	

* Mean values for 20 rats.
† Standard deviation of the mean.
‡ $P < 0·01$ that differences between group killed by decapitation and group killed by immersion (Table 1) are due to chance.
§ $P < 0·02$ that difference between group killed by decapitation and group killed by immersion (Table 1) is due to chance.

Homogenization of the tissue with a glass homogenizer did not improve the recoveries of adenine nucleotides and CP. Extractions were about as efficient when either centrifuge tubes or homogenizing tubes were used, and because of the ease of washing and centrifuging, centrifuge tubes were employed in most experiments.

Control animals. Decapitation procedure. The levels of ATP and CP in brains of animals killed by decapitation are considerably lower than in control animals directly immersed in liquid oxygen. In comparison with the group killed by immersion, levels of AMP and ADP are higher in the decapitated group. There is no significant correlation between any of the adenine nucleotides or CP, and size of brain sample in this group of animals (Table 2).

Animals anæsthetized with ether. The levels of adenine nucleotide compounds in this group are not different from those of the control animals. CP levels, on the other

hand, are significantly greater in the anæsthetized animals. Though the number of animals is smaller, there is the same correlation of size of brain sample and levels of phosphate compounds as is observed in the control animals (Table 1).

Animals anæsthetized with pentobarbitone. The levels of phosphate compounds in this group are very similar to those of animals anæsthetized with ether. Only the CP level is significantly higher than that of the control group. As with the other groups killed by immersion, there is a correlation between the several phosphate compounds and the size of the brain sample (Table 1).

Enzyme studies. AMP is relatively slowly hydrolysed by brain homogenates, while ADP is much more rapidly attacked. The data reported give no information on whether the ADP is destroyed predominantly by direct hydrolysis, or indirectly through the adenylate kinase reaction. The phosphate accumulating in the medium rapidly exceeds the theoretical amount expected from the conversion of half the ADP to ATP and the subsequent splitting of the terminal phosphate from the latter compound, and it is probable that direct hydrolysis of ADP is occurring to a large extent. ATP is hydrolysed most rapidly, at a rate almost twice that of ADP. CP, in the absence of adenine nucleotide, is dephosphorylated at a very slow rate. In the presence of any of the adenine nucleotides, creatine phosphokinase activity is markedly increased. The formation of free creatine is most rapid in the presence of the diphosphate. Associated with the formation of free creatine is the rapid appearance of inorganic phosphate. In the presence of CP, ATP is hydrolysed more rapidly than in its absence. ATP is hydrolysed fairly rapidly even at low temperatures. At 4°, the rate of hydrolysis is approximately one-seventh that at 37° (Table 3).

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DISCUSSION

Changes in levels of brain labile phosphate compounds are so rapid and so easily induced that it is not surprising to find considerable differences reported in the literature of the 'normal resting', values even when the immersion procedures are employed. True variations among species may also contribute to the discrepancies. KRATZING and NARAYANASWAMI (1953), using guinea pigs, found ATP levels somewhat higher than, and CP levels comparable to, those reported in the present paper, but found no ADP present. THORN *et al.* (1955) and THORN *et al.* (1958), using rabbits, obtained ATP levels and CP levels somewhat higher than either those of KRATZING and NARAYANASWAMI or those presented here, and ADP levels somewhat lower than those presented here. THORN *et al.* employed a labile phosphorus procedure to determine CP, a method which is likely to give higher results than more specific methods (ENNOR and ROSENBERG, 1952). ALBAUM, TEPPERMAN, and BODANSKY (1946) reported comparable levels of ATP and slightly higher levels of ADP in rat brains than are reported here. LEPAGE (1946*a*, *b*), also using rats, found a much higher proportion of AMP and ADP and less ATP than the present results show. DÖRING and GERLACH (1957) and GERLACH, DÖRING, and FLECKENSTEIN (1958), presented values in rat brain comparable to those obtained in the small brain samples of this investigation (see below).

The extreme rapidity of breakdown of cerebral labile phosphates is evident when the data from rats killed by immersion are compared with those from rats killed by decapitation. More marked destruction of these compounds appears to occur with rats than with guinea pigs subjected to the same procedure (KRATZING and

NARAYANASWAMI, 1953). This rapid breakdown may be attributable to two factors: (1) the massive stimulation of the central nervous system as a result of the severing of the spinal cord; and (2) the breakdown of labile phosphates during the 10–15 sec necessary to freeze the brain.

Brief electrical stimulation of cerebral slices (HEALD, 1954) or of the brains of intact rats (DAWSON and RICHTER, 1950), or short periods of ischæmia or anoxia

TABLE 3.—RATES OF BREAKDOWN OF LABILE PHOSPHATE COMPOUNDS BY BRAIN HOMOGENATES

Substrate	μmoles P liberated per g tissue per sec	μmoles creatine liberated per g tissue per sec
ATP (19)*	0·75	—
S.D.†	0·06	
ADP (8)	0·42	—
S.D.	0·07	
AMP (8)	0·10	—
S.D.	0·01	
CP (2)	0·02	0·02
ATP + CP (6)	0·95	0·68
S.D.	0·13	0·06
ADP + CP (5)	0·51	0·97
S.D.	0·05	0·08
ATP 4° (8)	0·11	—
S.D.	0·02	

* Numbers of separate determinations of enzymic activity are in parentheses.
† S.D. = Standard deviation of the mean.

(THORN *et al.*, 1955, 1958) results in marked decreases in levels of brain labile phosphate compounds. Thus, there is much evidence to attribute at least part of the decreases in labile phosphates to the violent stimulation of decapitation. On the other hand, the freezing of the brain takes several seconds, and apparently the interior of the tissue is somewhat insulated by the more exterior layers (RICHTER and DAWSON, 1948). Conceivably, postmortem breakdown of nucleotides and CP could be occurring during the freezing process.

DÖRING and GERLACH (1957) studied levels of labile phosphate compounds in brains of anæsthetized rats killed by decapitation, and unanæsthetized rats killed by immersion in liquid oxygen, and found comparable levels of ATP, ADP, AMP, and CP in both groups. In unanæsthetized rats, decapitation led to a marked fall in ATP and CP. These results suggest that the massive stimulation of decapitation is the major factor contributing to the rapid breakdown of labile phosphates in brain.

In addition to the breakdown before freezing, the extraction process may result in some loss of labile phosphate compounds. Two factors which must be considered

here are: (1) breakdown during extraction; and (2) incomplete extraction from the frozen tissue.

The data presented indicate that there may be difficulty in completely extracting ATP, CP, and perhaps ADP unless the volume of extracting fluid is at least ten times the tissue sample size. This is true in spite of performing two subsequent extractions of the protein precipitant. If the volume of extracting fluid is kept constant, and the size of brain sample is varied, an inverse linear relationship between the concentration of the labile phosphate compounds and the brain sample size is obtained. The slope of the line for ATP is $-0{\cdot}63$ μmole/g; that for CP is $-1{\cdot}34$ μmole/g. If these lines are extrapolated to zero sample weight the values for ATP and CP are 1·69 μmole/g and 2·93 μmole/g. The regression line for ADP has a slight negative slope ($-0{\cdot}10$ μmole/g), but it does not differ significantly from zero. When AMP brain concentration is plotted against brain sample size, the regression line has a positive slope of 0·28. Extrapolation of this line to zero sample size yields a value for AMP of 0·20 μmole/g.

The positive slope of the AMP regression line suggests strongly that some breakdown of ATP and ADP to AMP is occurring while the brain is in the frozen state and before the acid protein precipitant has completely denatured the brain enzymes involved in adenine nucleotide dephosphorylation. The larger the brain sample, the more difficult it was to break the ice crust formed at the interface of tissue and acid when the frozen tissue was poured into the tube containing perchloric acid. Some breakdown of adenine nucleotides must be occurring at this stage, and it proceeds over a longer time with the larger brain samples. Though one would not expect much enzymic activity under these circumstances, the extremely great activity of these enzymes in brain tissue, the relatively low temperature coefficient of activity for ATPase (DAWSON and RICHTER, 1950) (Table 3), and the relative stability of adenylate kinase in acid (KALCKAR, 1947) are three factors which favour this explanation.

Both total creatine and total adenine nucleotide concentrations bear similar inverse relationships to brain sample size as do CP and ATP respectively, although the magnitude of these relationships is slightly less in the former instances. This strongly indicates that the major factor in obtaining low recoveries of ATP and CP from large samples is incomplete extraction. There seems to be no difficulty in extracting free creatine, since no significant relationship is found between the concentration of this material in brain and sample size. It appears that extraction is essentially complete if the successive ratios of volume of extracting acid to brain sample size are at least 10:1, 5:1, and 5:1.

ATPase and creatine phosphokinase are both extremely active in brain tissue (DUBOIS and POTTER, 1943; GORE, 1952; NARAYANASWAMI, 1952; Table 3), and it is not surprising that the assay of *in vivo* levels of brain labile phosphate compounds has been exceedingly difficult. Within 2–3 sec, all the ATP, ADP, and CP present within the brain could be enzymically degraded at 37°. Rapid destruction of ATP can also proceed at lower temperatures (Table 3; DAWSON and RICHTER, 1950). The acceleration of ATPase activity in the presence of CP suggests that, in brain, where CP levels are high, the ATPase activity may be potentially greater than that suggested by studies of ATPase activity when ATP alone is used as substrate.

Animals anæsthetized with either ether or pentobarbitone have the same brain levels of AMP, ADP, and ATP as those of control animals. These results are in agreement with those of DÖRING and GERLACH (1957), but in contrast to those of

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LEPAGE (1946*a*, *b*), who found that ATP was the predominant nucleotide in pentobarbitone-anæsthetized rats, whereas AMP and ADP constituted the largest proportion of adenine nucleotides in control animals. STONE (1940) found ATP to be only slightly greater in anæsthetized mice than in controls.

CP concentrations, on the other hand, are significantly higher in both groups of anæsthetized animals than in control animals, a finding which corroborates previous studies (LEPAGE, 1946*a*, *b*; STONE, 1940). The data suggest that the brain levels of adenine nucleotides presented here closely reflect the *in vivo* levels of these compounds. Presumably, the waking, quiet state and the trauma associated with immersion in liquid oxygen do require the expenditure of ATP and result in the depletion of some of the CP stores to regenerate the utilized ATP. This resynthesis is apparently performed with a rapidity sufficient to reconstitute the adenine nucleotides. Anæsthetized animals presumably utilize labile phosphates at a lower rate than conscious animals, and this is reflected in a larger store of CP. However, the adenine nucleotides are apparently already phosphorylated to an optimal degree, since the higher levels of CP are not being more rapidly utilized in the phosphorylation of AMP and ADP.

The mean values of free creatine in the control and anæsthetized groups of rats were essentially identical (6·7 μmole/g). The increased creatine phosphate in the two anæsthetized groups was associated with an equal rise in the total creatine concentration of the brain (pentobarbital group vs. controls, $P < 0{\cdot}05$; ether group vs. controls, $P < 0{\cdot}10$), and suggests that during anæsthesia the brain synthesizes or takes up an amount of creatine sufficient to maintain the levels of this substance in the unesterified form. In contradistinction, the brief stimulus of decapitation results in a fall in CP and a proportionate rise in free creatine concentration in brain. The latter observation is in agreement with that of ISSELHARD, IRMSCHER, and THORN (1959), who observed that free creatine rose and CP fell during periods of acute ischæmia or anoxia.

The data suggest that the primary pharmacological action of ether and pentobarbitone cannot be depression of oxidative metabolism and depression of ATP synthesis (JOHNSON and QUASTEL, 1953), or uncoupling of oxidative phosphorylation (BAIN, 1952). It appears that these agents directly or indirectly interfere with the utilization of ATP rather than with its synthesis (MCILWAIN, 1953). Conceivably, however, they might selectively inhibit ATP synthesis in certain areas of the brain, resulting in a depression of brain function locally, and secondary depression of overall brain function with accumulation of labile phosphate compounds elsewhere (MCILWAIN, 1959). This would be reflected in elevations of the concentrations of labile phosphate compounds if the whole brain is analysed, and could be one explanation for the results obtained in the present study.

SUMMARY

Adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, and creatine phosphate levels of rat brain were studied under a variety of circumstances. In unanaesthetized animals killed by immersion in liquid oxygen, levels of adenosine monophosphate, adenosine diphosphate, adenosine triphosphate and creatine phosphate were 0·42 μmole/g, 0·66 μmole/g, 1·22 μmole/g, and 2·02 μmole/g, respectively. Adenosine triphosphate and creatine phosphate are incompletely extracted from brain by perchloric acid unless sufficiently large ratios of volume of acid to sample size

are used. During the extraction of the frozen tissue, some breakdown of labile phosphate compounds is apparently occurring, in spite of efforts to avoid this. Decapitation before immersion of the severed head in liquid oxygen results in much lower levels of brain adenosine triphosphate and creatine phosphate and higher levels of adenosine monophosphate, owing to the rapid breakdown of the labile phosphate compounds during decapitation and prior to complete freezing of the brain. Anæsthesia with either ether or pentobarbitone before immersion of the whole animal in liquid oxygen results in the recovery of higher brain levels of creatine phosphate but no change in levels of adenine nucleotides as compared with those of similarly handled control animals. The activities of 'adenosine triphosphatases' and creatine phosphokinase in rat brain are sufficiently great to be capable of destroying all the adenosine triphosphate and creatine phosphate present in brain in a few seconds.

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ULTRACENTRIFUGAL FRACTIONATION OF NERVE TISSUE

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CHEMICAL data relating to myelin have been obtained exclusively from studies of whole nerve tissue. Comparative studies of myelinated and unmyelinated nervous tissue have permitted deductions relating to myelin composition but some uncertainties persist which limit the value of the chemical data in relation to structural studies. The structural data obtained by polarized light, X-ray diffraction and electron microscopy (ENGSTRÖM and FINEAN, 1958) relate specifically to the myelin component and therefore the interpretation of the data in molecular terms demands a precise knowledge of the chemical composition of the myelin sheath. This knowledge can only be obtained unequivocally by chemical analysis of the myelin sheath itself and as yet no reliable method for its isolation in quantities sufficient for chemical analysis has been described. Myelin was separated from whole nerve tissue by ultracentrifugation by FERNÁNDEZ-MORÁN (1952), but the microquantities isolated were insufficient for chemical analysis by methods available at that time.

The work to be described in this paper is a preliminary attempt to isolate myelin in quantities sufficient for chemical analysis. The method adopted was differential centrifugation of homogenized nerve tissue. It was anticipated that the homogenization would modify the structure to some degree and an attempt was made to study the extent of modification using X-ray diffraction methods and also by examination of the resulting preparations by electron microscopy.

MATERIAL AND METHODS

Bovine optic nerve was chosen as it was available in large quantities and had previously been selected as an example of central nerve material suitable for X-ray diffraction studies (TEALE, 1950).

Two series of experiments were made. The suspending medium used in the first series was 20% sodium chloride solution; in the second series it was 0·88 M-sucrose solution. The dilutions required to provide a range of densities suitable for the fractionation were determined in preliminary experiments.

Experiments using saline as suspending medium. The fractionation procedure is summarised in Fig. 2. All operations were carried out at 4°. The nerves were first dissected free from their connective tissue sheaths and were then homogenized in 20% sodium chloride solution. The homogenization conditions were found to be critical for constancy of fractionation and, therefore, were standardized as far as possible. The homogenization was carried out in two stages. The initial stage was a rough homogenization in a perspex Potter-Elvehjem homogenizer having a wide gap between piston and barrel. The resulting homogenate was then transferred to a similar homogenizer having a much smaller gap between piston and barrel and rehomogenized. In each case the piston of the homogenizer was rotated at 600 rev/min in a drill press. Incompletely homogenized material was removed by centrifuging at 1900 **g** for 20 min. It was found that this material settled to the bottom of the tube whilst the homogenized material was packed in a floating layer. This floating layer was isolated by decantation and rehomogenized in 20% sodium chloride solution. The homogenate was made up to 100 ml and then diluted by adding 50 ml of distilled water. It was then thoroughly dispersed and centrifuged at 20,000 **g** for 1 hr. After this centrifugation two discrete fractions were obtained,the first consisting of particles having a density greater than that of the suspending medium and therefore

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settling in the gravitational field and the second being particles of density less than that of the suspending medium and therefore moving in the opposite direction to the gravitational field and forming a well packed floating fraction (see Fig. 1). The sediment (Fraction 1) was dialysed against running water to remove sodium chloride and was then freeze-dried. The fractionation of the floating layer was repeated twice (Fig. 2) giving a settled fraction at each stage (Fractions 2 and 3) and a final

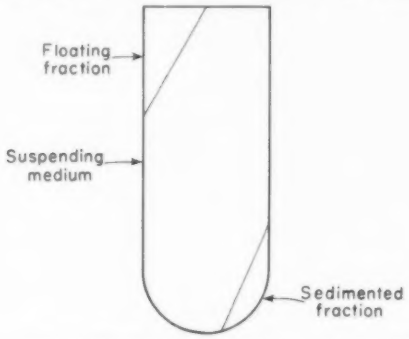

FIG. 1.—Typical distribution of fractions after centrifugation.

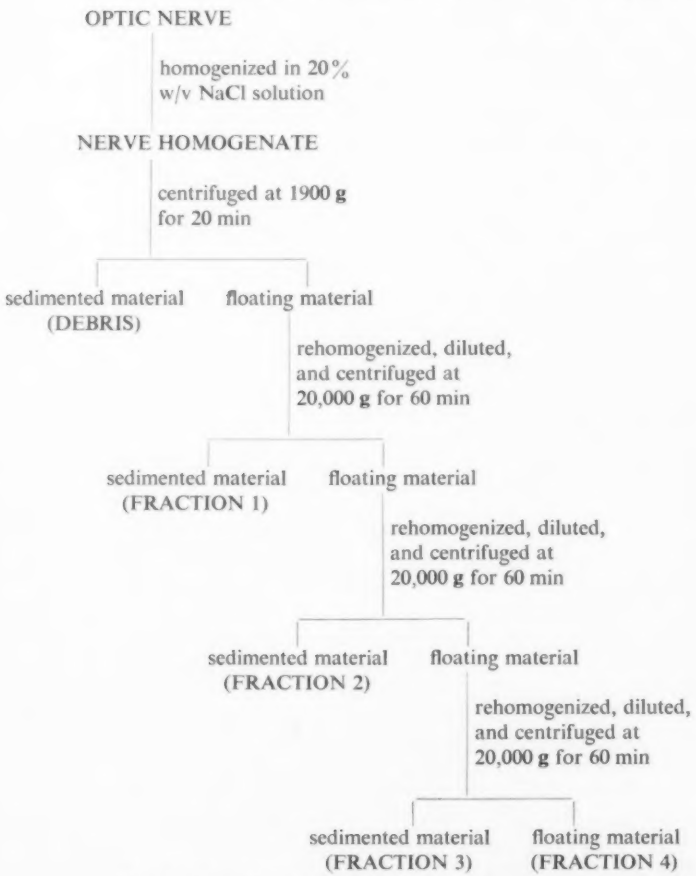
OPTIC NERVE
homogenized in 20% w/v NaCl solution
NERVE HOMOGENATE
centrifuged at 1900 g for 20 min
sedimented material (DEBRIS)
floating material
rehomogenized, diluted, and centrifuged at 20,000 g for 60 min
sedimented material (FRACTION 1)
floating material
rehomogenized, diluted, and centrifuged at 20,000 g for 60 min
sedimented material (FRACTION 2)
floating material
rehomogenized, diluted, and centrifuged at 20,000 g for 60 min
sedimented material (FRACTION 3)
floating material (FRACTION 4)

FIG. 2.—Fractionation procedure using saline as suspending medium.

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floating layer (Fraction 4). All four dialysed and freeze-dried fractions were white powders. In some experiments a further fraction in the form of a light brown powder was obtained from the supernatants of all stages which had been pooled, dialysed to remove salt, and freeze-dried.

Experiments using sucrose as a suspending medium. The preliminary homogenization was carried out in 0·88 M-sucrose solution made up in 0·9% sodium chloride solution. The method used was essentially similar to that already described, and a summary of the fractionation procedure is given in Fig. 3. In these experiments five fractions were obtained. The material remaining in the pooled supernatants was not isolated.

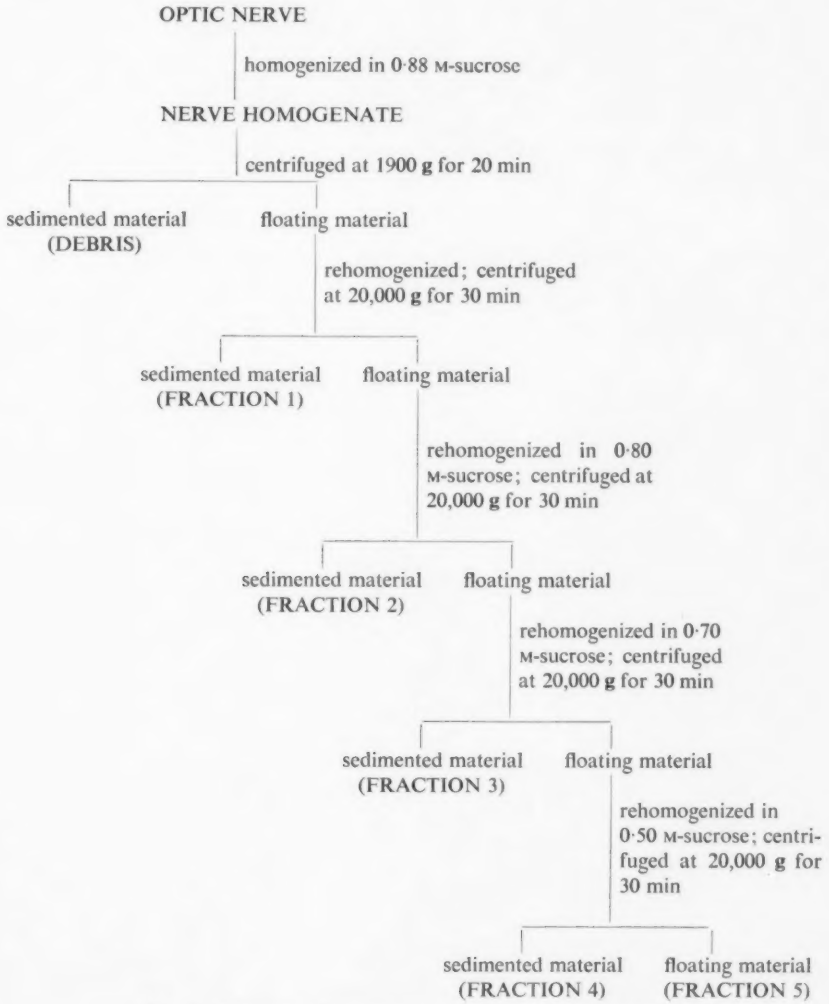
OPTIC NERVE
homogenized in 0·88 M-sucrose
NERVE HOMOGENATE
centrifuged at 1900 g for 20 min
sedimented material (DEBRIS)
floating material
rehomogenized; centrifuged at 20,000 g for 30 min
sedimented material (FRACTION 1)
floating material
rehomogenized in 0·80 M-sucrose; centrifuged at 20,000 g for 30 min
sedimented material (FRACTION 2)
floating material
rehomogenized in 0·70 M-sucrose; centrifuged at 20,000 g for 30 min
sedimented material (FRACTION 3)
floating material
rehomogenized in 0·50 M-sucrose; centrifuged at 20,000 g for 30 min
sedimented material (FRACTION 4)
floating material (FRACTION 5)

FIG. 3.—Fractionation procedure using sucrose as suspending medium.

Analysis of the fractions. After weighing, each of the fractions was exhaustively extracted with 2:1 (v/v) chloroform:methanol. The extracts were made up to known volumes, and portions were examined quantitatively for phosphorus (KING, 1932), cholesterol (SPERRY and WEBB, 1950), and cerebroside (SVENNERHOLM, 1956). The insoluble material (assumed to be protein) was weighed and its nitrogen content was determined using the micro-Kjeldahl method.

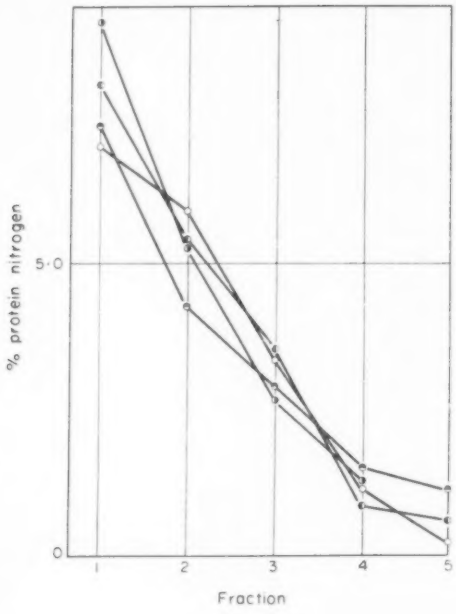

FIG. 4.

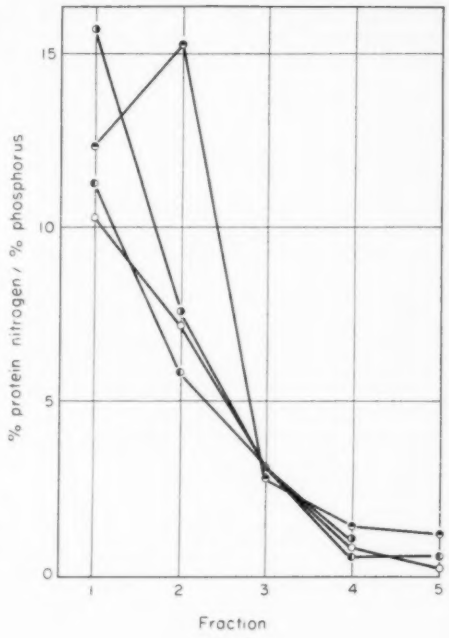

FIG. 5.

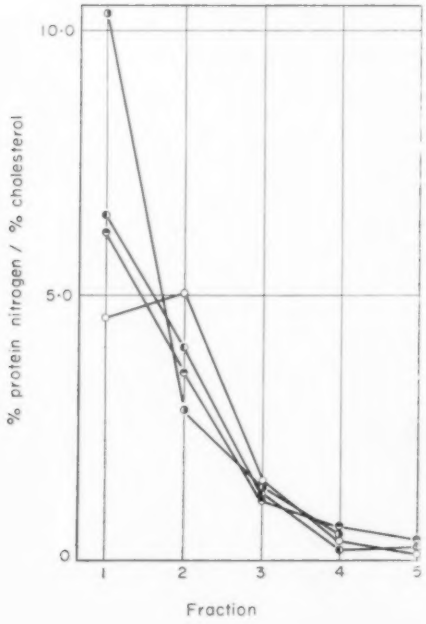

FIG. 6.

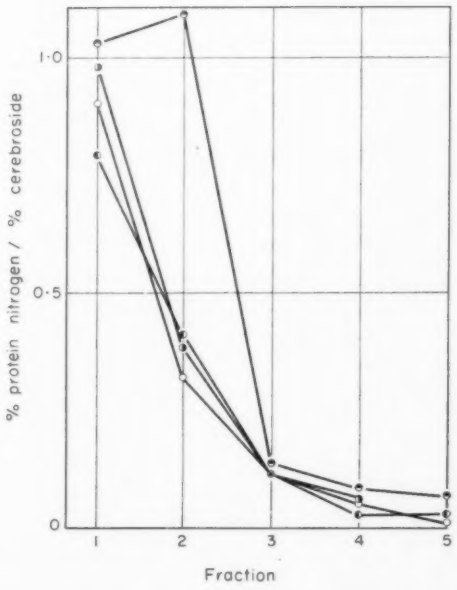

FIG. 7.

FIGS. 4–7.—Experiments using sucrose solutions as suspending media. Fig. 4 shows the percentage protein nitrogen insoluble in 2:1 (v/v) chloroform:methanol in each fraction. Figs. 5–7 show the ratios of percentage protein insoluble in 2:1 (v/v) chloroform:methanol to the percentage of some lipid components soluble in the same solvent. In Expt. 4 Fraction 5 was not obtained in quantities sufficient for chemical analysis.

Expt. 1○ Expt. 2◓ Expt. 3◐ Expt. 4◑

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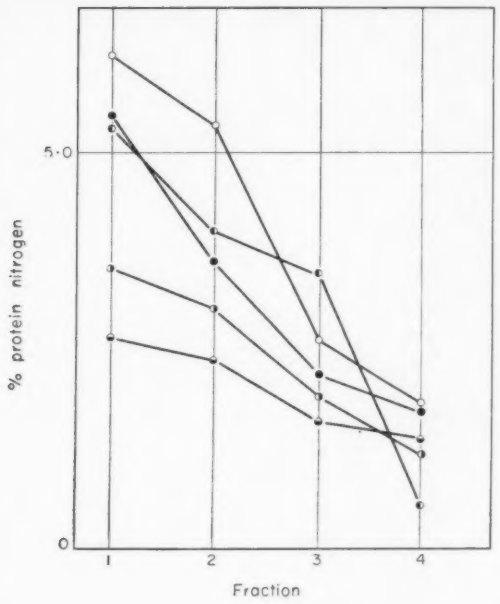

FIG. 8.

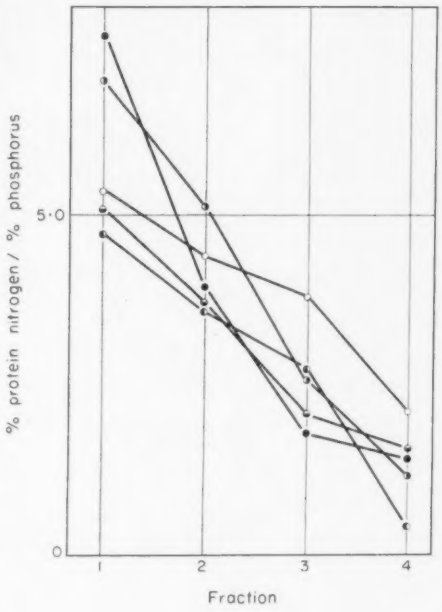

FIG. 9.

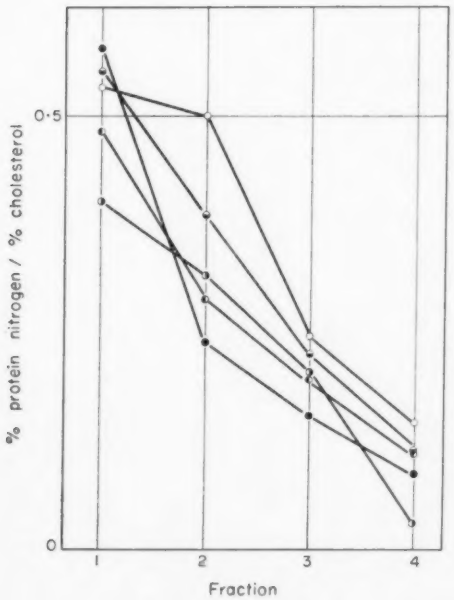

FIG. 10.

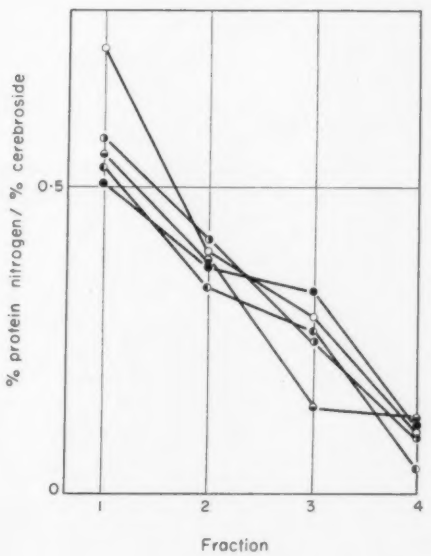

FIG. 11.

FIGS. 8–11.—Experiments using saline solutions as suspending media. Fig. 8 shows the percentage protein nitrogen insoluble in 2:1 (v/v) chloroform:methanol in each fraction. Fig. 9–11 show the ratios of percentage protein insoluble in 2:1 (v/v) chloroform:methanol to the percentage of some lipid components soluble in the same solvent.

Expt. 5○ Expt. 6● Expt. 7◐ Expt. 8◑ Expt. 9◒

Examination of the fractions by electron microscopy and X-ray diffraction. Before freeze-drying, samples were removed and transferred to osmic acid for fixing before embedding in Araldite and thin-sectioning for examination by electron microscopy. Samples were also removed and sealed in thin-walled glass tubes for examination by X-ray diffraction. The low angle diffraction patterns were then obtained using high resolution vacuum cameras previously described (FINEAN, 1956).

TABLE 1.—EXPERIMENTS USING SUCROSE SOLUTIONS AS SUSPENDING MEDIA

Experiment	Molecular ratio of Fraction 5		
	Phospholipid	Cholesterol	Cerebroside
1	1·0	1·6	0·6
2	1·0	1·8	0·5
3	1·0	1·7	1·1
4*	1·0	1·5	0·5

* Fraction 4

TABLE 2.—EXPERIMENTS USING SALINE SOLUTIONS AS SUSPENDING MEDIA

Experiment	Molecular ratio of Fraction 4		
	Phospholipid	Cholesterol	Cerebroside
5	1·0	1·2	0·6
6	1·0	1·0	0·4
7	1·0	1·1	0·6
8	1·0	1·1	0·8
9	1·0	0·9	0·45

RESULTS

Chemical analysis

Results of four experiments using sucrose as the suspending medium and of five experiments using sodium chloride as the suspending medium are given.

Figs. 4 and 8 show the percentage of protein nitrogen insoluble in 2:1 (v/v) chloroform to methanol in each fraction. The ratios of this nitrogen value to the individual lipid components present in the fractions are shown in Figs. 5 to 7 and 9 to 11. The molecular ratios of phospholipid (phosphorus value $\times$ 26·5) to cholesterol to cerebroside in Fraction 5 (sucrose) and Fraction 4 (saline) are shown in Tables 1 and 2.

The composition of the material isolated from pooled supernatants in the fractionation in which saline was the suspending medium was found to be very similar to that reported by LEBARON and FOLCH (1959) for their 20% saline extracts of brain.

X-ray diffraction

The nerve tissue homogenized in either the sucrose or saline medium gave only two diffraction bands, an intense one at 68 Å and a much fainter one at 34 Å. All the subsequent homogenized fractions showed the 68 Å band but in some cases the 34 Å band was not detected. The diffraction was most intense in the case of the first floating fraction and was barely detectable in the first sedimented fraction. By comparison the diffraction pattern of optic nerve showed 80 Å and 40 Å bands which

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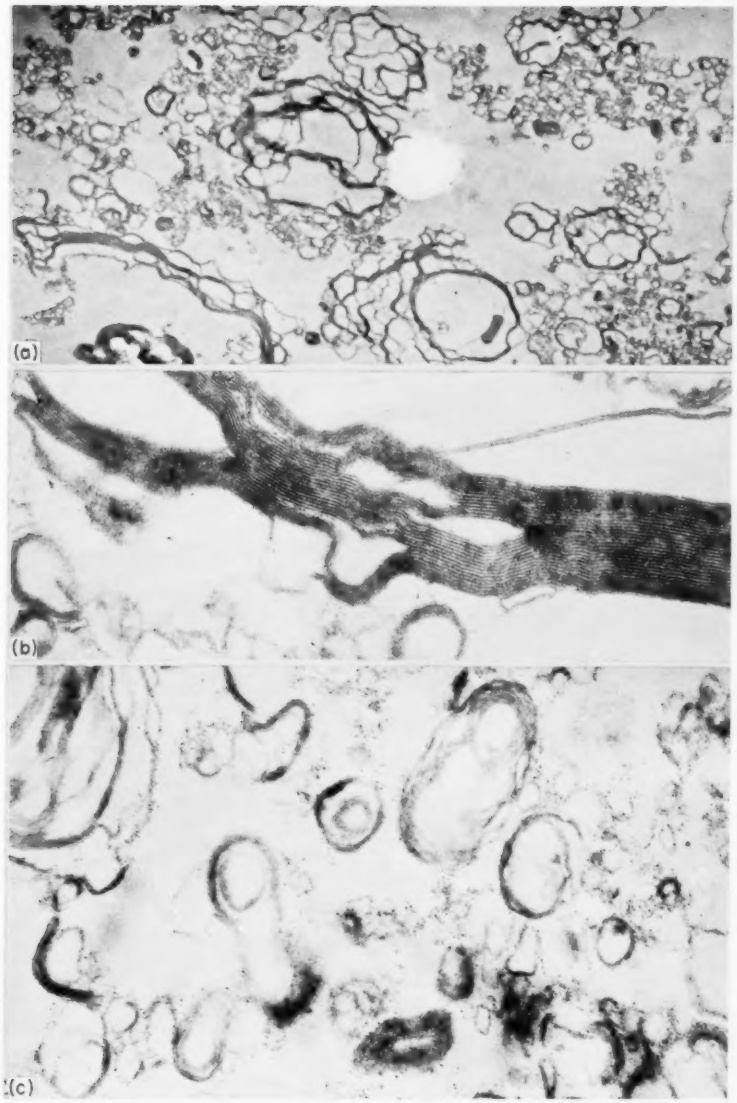

FIG. 12.—Electron micrographs of a fraction obtained from optic nerve homogenized in sucrose solution (*a*) segments of intact myelin and dispersed membranous network. ×8,000. (*b*) detail of myelin layering in a segment of intact myelin. ×64,000. (*c*) remnants of layering in the dispersed membranous material. ×32,000.

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are considered to be related (as second and fourth order diffractions) to a myelin unit measuring 160 Å (FINEAN, 1953). Consequently, if the 68 Å and 34 Å diffractions of homogenized tissue are also to be related to this myelin unit then a shrinkage of about 24 Å is assumed to have been produced by the homogenization.

Electron microscopy

All samples examined in the electron microscope showed segments of intact myelin sheath together with a meshwork of membranous material in which long strands and circular profiles could be distinguished (Fig. 12*a*). The smallness of the samples, inherent in the electron microscope technique, did not allow a quantitative assessment to be made of the relative proportions of these materials in the various fractions. Nevertheless the method did demonstrate that the final fractions contained a high proportion of myelin segments in which the myelin layers were of essentially normal appearance (Fig. 12*b*). Furthermore, where layering could be distinguished in the dispersed membranous material it was very similar to the myelin layering (Fig. 12*c*).

DISCUSSION

In both series of experiments there was a progressive decrease in the proportion of chloroform to methanol insoluble material in the fractions and corresponding increases in the proportions of lipids. Much of this insoluble material must have been protein derived from axon and connective tissue components but it could conceivably have included some non-lipid components of myelin. There were, however, many indications that most of the myelin had remained intact.

Electron micrographs provided a direct demonstration of a large proportion of intact myelin layering in the final fractions and failed to detect any of the finer (40 Å) layering characteristic of hydrated lipid systems (STOECKENIUS, 1959). These data also facilitate the interpretation of the X-ray diffraction results. The diffraction pattern recorded from all fractions was somewhat modified in comparison with that of the intact tissue, and considered alone could not have been distinguished unequivocally from that of emulsified lipid. However, the only components seen in the electron micrographs that seemed capable of producing well-defined diffractions were the segments of myelin sheath. Consequently the marked differences in the intensities of diffraction from settled and floating fractions in the first stage of fractionation can be taken as a clear indication that few of the myelin fragments were precipitated, but were concentrated in the floating fraction. The fact that the intensity of diffraction did not increase further as the fractions became richer in myelin can be explained in terms of a further dispersion of the myelin layers by the repeated homogenization.

The remarkable constancy of the molecular ratios among the lipid components would also be in keeping with the persistence of intact myelin in all fractions. The ratios established in the saline series correspond closely to those previously suggested for myelin from other chemical studies, but there is some discrepancy between this and the sucrose series which is not yet explained.

The combined data clearly indicate that at least a partial separation of intact myelin from other nerve components was achieved in both series of experiments.

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THE CONTENT OF AMINO ACIDS IN THE PROTEINS OF THE DEVELOPING NERVOUS SYSTEM OF THE GUINEA PIG—I

CEREBRAL WHITE MATTER

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IN THE course of the development of the nervous system profound biochemical changes occur along with differentiation of the structural components. The investigations undertaken aimed at correlating the morphological and biochemical phenomena, and indirectly at providing information about the chemical composition of some of the structural components of the nervous system.

Little is known about the changes of amino acid composition of the proteins of nervous tissue during maturation of the central nervous system or of their correlation with what is known about the myelination of nerve fibres from histological examination. Up to date reports on this subject are very scanty. TUSTANOWSKIJ (1938) found the content of arginine in proteins of the foetal markedly higher than in the postnatal brain. DOBRYNINA (1940) has shown that the content of tryptophan is higher and the content of cystine lower in the infant than in the adult brain. In the material examined by her there were no significant differences in the content of tyrosine, histidine, arginine or lysine at different ages.

Previous investigations were concerned with only a few amino acids, whereas the present paper reports changes in a number of amino acid in the proteins of the cerebral white matter of the guinea pig during development. The chemical determinations are compared with the histological demonstration of myelin and developing glial cells.

EXPERIMENTAL

Materials and methods. Investigations were made on a total of 70 guinea pigs of both sexes which were divided into 7 age-groups with 10 animals in each group: foetuses (8–9th week of uterine life), 3, 5, 9 and 12 days, 2 months and adults of 6–8 months. The animals were anaesthetised with ether, killed by bleeding and the brain was removed from the cranial vault as quickly as possible. The samples of cerebral tissue for chemical examination included the corpus callosum with its radiation, the white matter of the cerebral hemispheres, the cerebral peduncles, cerebellar hemispheres and the macroscopically visible white matter of the cerebral trunk. Samples were taken from the same topographical areas in the foetal specimens. Samples for histological examination were fixed in 15% (w/v) formalin. Slices were cut on a freezing microtome at 30 μ and stained by the myelin method of SPIELMEYER (1930). Further slices were cut at 20 μ and stained by the cresyl violet method of NISSL in the modification of SPIELMEYER (1930). The state of myelination in the early stages of development (foetuses, 3-and 5-day-old animals) was also determined in 5 μ paraffin slices by the osm-haematoxylin method of SCHULTZE (1906).

The samples for chemical examination were immediately weighed and lyophilized. The lyophilized material was dried to constant weight and analysed by paper chromatography.

Preparation of sample. Cerebral tissue (about 0·1 g) was weighed with accuracy to four places of decimals, ground, transferred to a centrifuge tube and extracted three times for 30 min on a shaker with 1 ml of acetone acidified with HCl. The acetone solution containing the free amino acids was removed by centrifugation (BOULANGER and BIZERTE, 1949; MCCOLLUM and RIDER, 1952). The denaturated proteins were hydrolysed with 0·5 ml 6 N-HCl or 0·5 ml 80% HCOOH in sealed tubes at 110° for 24 hr. The protein hydrolysate was centrifuged to remove any humin and taken to dryness in a vacuum evaporator. The dry residue was dissolved in acetone and dried in a vacuum evaporator. Acetone was added to the dry residue to give a 3% solution of hydrolysate (BLOCK, STRANGE and ZWEIG, 1952).

Chromatography. 10 μl of the 3% solution of hydrolysate were applied to Macherey-Nagel No. 61 paper. The following solvent systems were used:

1. Phenol saturated with sodium hydroxide–disodium phosphate buffer pH 12 (MCFARREN, 1951) and containing 0·04% 8-hydroxyquinoline (BLOCK, 1951) was used for determination of aspartic acid, glutamic acid, serine, glycine and threonine by ascending chromatography.

2. *n*-Butanol–glacial acetic acid–water (4:1:1 by volume) (REED, 1950) for determination of tyrosine, valine, phenylanine, leucine by ascending chromatography and of cystine, lysine, histidine and arginine by descending chromatography over 48 hr.

Quantitative determination of amino acids. The chromatograms were dried for 2 hr at room temperature and treated with a mixture of ethyl ether and acetone (1:1 v/v) to remove phenol. They were developed in a 0·1% acetone solution of ninhydrin and in an acetone solution of copper nitrate acidified with nitric acid. After elution with methanol the colour complexes of free amino acid groups were measured at 530 mμ using the Colemann-Junior spectrophotometer. Standard solutions of the amino acids mentioned above were used for comparison. The remaining amino acids were not examined.

The results of the investigations are given in g/100 g dry tissue as in early ontogenesis great changes were found in the water content of the nervous tissue of guinea pigs (WENDER and HIEROWSKI, 1960).

RESULTS

In describing the morphological differentiation and staining of myelin in white matter, only the basic phenomena, which may reflect significantly on the chemical composition of the nervous tissue, are considered.

In stained sections of the foetal brain of guinea pigs (8–9th week of uterine life), the myelinated nerve fibres both of the cerebral trunk and the cerebral hemispheres were not yet visible. The slides stained by the Nissl method showed a few scattered and immature glial cells, only partially aligned along the unmyelinated tracts in the white matter. Sections from the next group, animals in the first period of extra-uterine life (3-day-old), demonstrated the complete myelination of the nerve fibres in the cerebral trunk and in the cerebellar white matter (Fig. 1). The nerve fibres of the cerebral hemispheres and of the corpus callosum were still only weakly stained by the Spielmeyer and the Schultze methods (Fig. 2). Sections of the white matter stained by the Nissl method contained large numbers of cell nuclei which corresponded apparently to oligodendroblasts, oligodendrodrocytes and astroblasts. The arrangement of glial cells in long cordons along the total and partially myelinated nerve fibres is characteristic of this period of development. The sections of brain from the 5-day-old animals were indistinguishable from 3-day-old animals.

The slides prepared from animals aged 9 days and 12 days (Fig. 3), and stained by the Spielmeyer method showed myelination of the nerve fibres in the philogenetically younger as well as the older parts of the brain and were indistinguishable from adult brain (6–8 months of life). Sections stained by the Nissl method showed greater numbers of glial cells but were less mature than in white matter of adult brain. The

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FIG. 1.—3-day-old guinea pig. Sagittal section of the cerebral trunk and of the cerebellum shows the complete myelination of the nerve fibres. Stained by the method of SPIELMEYER. × 10.

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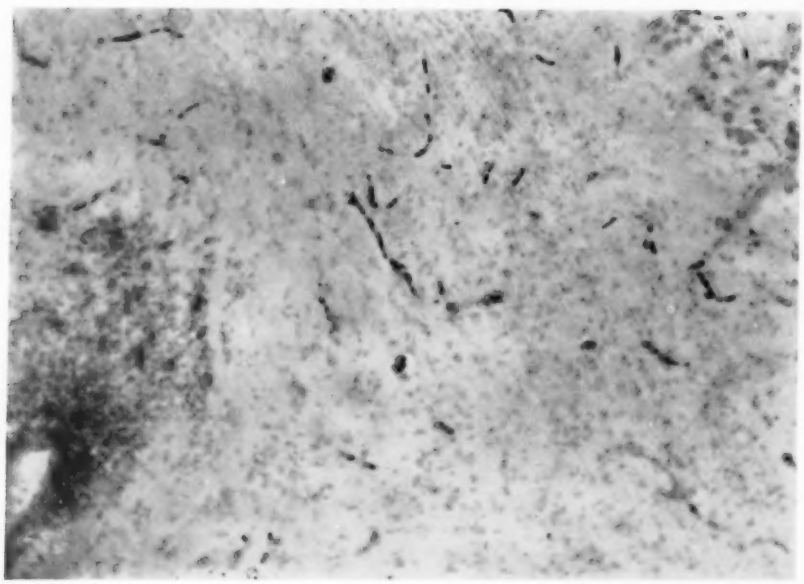

FIG. 2.—3-day-old guinea pig. Cerebral white matter shows only few and weakly stained nerve fibres. Stained by the method of SPIELMEYER. × 120.

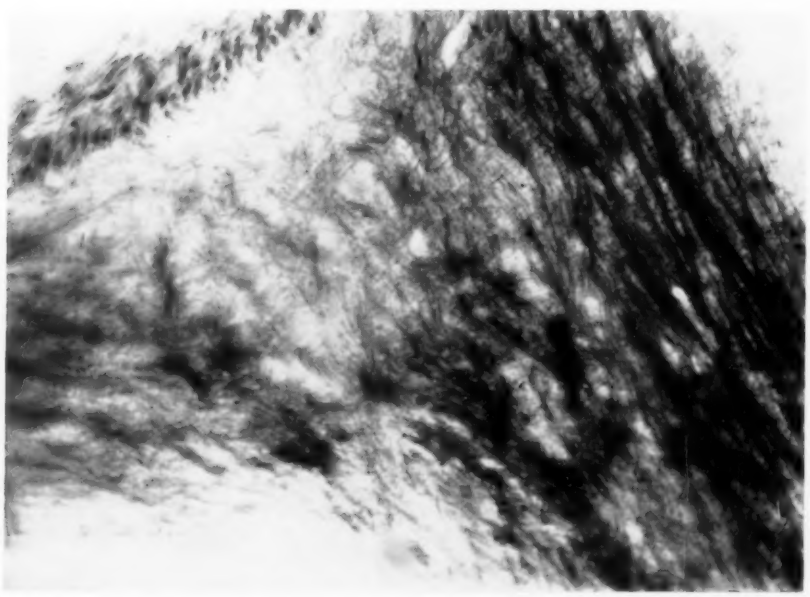

FIG. 3.—9-day-old guinea pig. Cerebral white matter shows myelination of the nerve fibres. Stained by the method of SPIELMEYER. × 120.

TABLE 1.—CONTENT OF AMINO ACIDS IN THE PROTEIN OF CEREBRAL WHITE MATTER OF GUINEA PIGS DURING DEVELOPMENT

No. of animals in each group: 10. The results are given as mean (g/100 g dry tissue) ± standard error.

	Foetuses (8–9th week of gestation)	Age					
		3 days	5 days	9 days	12 days	2 months	8–10 months
Cystine	0·33 ± 0·04	0·43 ± 0·04	0·42 ± 0·08	0·33 ± 0·07	0·43 ± 0·07	0·44 ± 0·07	0·53 ± 0·09
Lysine	1·54 ± 0·07	1·48 ± 0·18	1·54 ± 0·19	1·79 ± 0·10	1·74 ± 0·37	1·83 ± 0·20	1·84 ± 0·11
Histidine	2·82 ± 0·17	2·13 ± 0·05	2·14 ± 0·22	1·03 ± 0·14	1·24 ± 0·14	0·63 ± 0·06	0·63 ± 0·06
Aspartic acid	1·83 ± 0·12	2·23 ± 0·12	2·29 ± 0·17	2·54 ± 0·25	2·53 ± 0·29	2·45 ± 0·19	2·44 ± 0·14
Glutamic acid	0·84 ± 0·08	2·13 ± 0·15	2·13 ± 0·16	2·46 ± 0·11	2·54 ± 0·30	3·30 ± 0·33	2·93 ± 0·21
Threonine	0·54 ± 0·05	0·91 ± 0·14	0·94 ± 0·17	0·74 ± 0·03	0·73 ± 0·15	0·85 ± 0·10	0·93 ± 0·12
Serine	3·23 ± 0·16	1·32 ± 0·13	1·42 ± 0·15	1·15 ± 0·10	1·03 ± 0·13	0·84 ± 0·30	0·83 ± 0·09
Arginine	0·54 ± 0·05	0·85 ± 0·04	1·16 ± 0·12	0·94 ± 0·09	0·84 ± 0·14	1·13 ± 0·10	1·12 ± 0·14
Glycine	1·04 ± 0·14	1·15 ± 0·04	1·13 ± 0·14	1·34 ± 0·08	1·33 ± 0·13	1·24 ± 0·16	1·24 ± 0·15
Tyrosine	0·45 ± 0·07	1·03 ± 0·13	1·04 ± 0·13	1·13 ± 0·03	1·15 ± 0·15	1·30 ± 0·17	1·04 ± 0·13
Valine	4·16 ± 0·30	2·14 ± 0·14	2·12 ± 0·20	1·55 ± 0·16	1·54 ± 0·19	1·43 ± 0·14	1·53 ± 0·19
Phenylalanine	0·85 ± 0·07	2·04 ± 0·21	2·09 ± 0·16	2·43 ± 0·28	2·33 ± 0·22	2·23 ± 0·14	2·33 ± 0·15
Leucine	2·13 ± 0·18	3·62 ± 0·26	3·65 ± 0·23	3·82 ± 0·27	3·83 ± 0·23	4·35 ± 0·30	3·93 ± 0·33
Water in g/100 g of fresh tissue	90·5 ± 0·5	82 ± 1·0	82 ± 0·5	81 ± 1·0	80 ± 1·0	72 ± 1·0	69 ± 1·0

number of glial cells in the cerebral white matter of 2-month-old and adult animals was markedly lower than in the period of myelination of the nerve fibres.

Considerable differences were found in the content of various amino acids in the proteins of cerebral white matter (Table 1) between foetal and 3-day-old animals. Even greater differences were found between foetal and adult animals. Values obtained from foetal white matter (expressed in terms of dry weight) show a greater content of histidine, serine and valine, but a smaller content of glutamic acid, arginine, tyrosine, phenylalanine and leucine. The differences were statistically significant. The content of cystine, lysine, aspartic acid, threonine and glycine did not differ significantly in foetal tissue from values found in the period of extra-uterine development.

The content of the amino acids in proteins from the 3- and 5-day-old animals was strikingly similar. Greater differences were found between 5- and 9-day-old animals but these were not statistically significant. An exception was the decrease of histidine content by 52 per cent. The results from 9- and 12-day-old animals were similar and the differences between these groups and 2-month-old animals and adults (6–8 months of life) were very small. There was a further decrease in the histidine content between 12 days and 2 months of life.

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DISCUSSION

In the first few days of extra-uterine life marked changes occurred in the content of some amino acids in the proteins of the white matter of the guinea pig brain. The content of glutamic acid, arginine, tyrosine, phenylalanine and leucine increased and the content of histidine, serine and valine decreased. This period was also the decisive period of myelination and there was a great increase in the number of glial cells, which are involved in this process. The coincidence of the changes in the content of some amino acids and myelination of nerve fibres suggests that these phenomena are connected. If they are, it is possible to assume that the proteins, which together with lipids form the basic structure of myelin sheath, contain more glutamic acid, arginine, tyrosine, phenylalanine and leucine and less histidine, serine and valine than proteins of other structural components of cerebral white matter. In the period of later development, despite a decrease in the number of glial cells in the cerebral white matter, the content of the amino acids did not return to the initial value showing clearly that the changes in the amino acids content during early development could not be due to the increase in the number of glial cells during myelination.

SUMMARY

The content of some amino acids in the proteins of cerebral white matter was examined in the developing nervous system of guinea pigs. The chemical examinations were correlated with the development of glial cells and myelination as observed histologically. The data found have led to the following conclusions:

1. The proteins of the foetal white matter of guinea pigs contain more histidine, serine and valine and less glutamic acid, arginine, tyrosine, phenylalanine and leucine than after birth.

2. The onset of histologically detectable myelination parallels the increase in the content of glutamic acid, arginine, histidine, serine and valine in the proteins of cerebral white matter.

3. The amino acid content of the proteins of the myelin sheath differs from that of proteins of other structural components of cerebral white matter in that they contain more glutamic acid, arginine, tyrosine, phenylalanine and leucine and less histidine, serine and valine.

4. The content of histidine in cerebral white matter decreases markedly during extra-uterine development.

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TRYPTOPHAN METABOLISM IN SCHIZOPHRENIC PATIENTS

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SEVERAL facts suggest the existence of a definite relation between the metabolism of aromatic amino acids and mental disease, and between indole compounds and schizophrenia. In recent years, much research has been carried out in the attempt to shed light on this problem. BOGOCH (1957) noted that the administration to 23 schizophrenic patients of a diet deficient in aromatic amino acids for three weeks had marked effects; in 60 per cent of the treated cases the clinical condition deteriorated, while no improvement was noted in the others; these results indicate that the diet tested not only had no therapeutic effect but was apparently detrimental.

Concerning tryptophan metabolism (by the kynurenine pathway) ZELLER *et al.* (1957), ZELLER (1958) and LAUER *et al.* (1958) reported that tryptophan (50 mg/kg) administered to 24 schizophrenic patients caused an increase in xanthurenic acid excretion which was significantly higher than that in normal controls: the difference between patients and controls disappeared in 5 weeks when iproniazid (Marsilid) was administered together with tryptophan.

While V. M. BUSCAINO (1957) maintained that indole derivatives play an important role in the causation of schizophrenia, G. A. BUSCAINO and STEFANACHI (1958) concluded from the analysis of the urines of schizophrenic and other patients that indole derivatives occur with varying frequencies in all cases examined and are not specifically related to schizophrenia.

Tryptophan metabolism was investigated in neurological diseases by VERGA (1951), Tinelli and Calvario (1955) and by Calvario (1958), who determined the xanthurenic acid excretion after administration of tryptophan (100 mg/kg). The greater amounts of xanthurenic acid eliminated by certain patients suggested an alteration of tryptophan metabolism, probably related to pyridoxine deficiency.

Concerning the other metabolic pathway of trytophan, studies have been made of the fraction of ingested tryptophan which is metabolized via serotonin to give 5-hydroxyindoleacetic acid. Although the excretion of this compound is generally normal in schizophrenic patients (BUSCAINO and STEFANACHI, 1958; FELDSTEIN *et al.*, 1958), it can be increased by the administration of large doses of tryptophan: ZELLER *et al.* (1957), ZELLER (1958) and LAUER *et al.* (1958) found that schizophrenic patients were unable to increase their elimination of 5-hydroxyindoleacetic acid after tryptophan ingestion while similarly treated non-psychotic controls doubled their excretion of this compound. BANERJEE and AGARWAL (1958), on the other hand, reported exactly the opposite: they found that schizophrenic patients doubled their excretion of this indole derivative while the controls did not. According to KOPIN (1959) schizophrenic patients do not differ from controls in their excretion of 5-hydroxyindoleacetic

acid either before or after oral tryptophan administration. He concluded that the elimination of the indole derivative depends on the amount of tryptophan in the diet and is not related to vitamin B_6 deficiency.

The difference between schizophrenic and non-schizophrenic subjects in their excretion of indole derivatives, and the role of these compounds in the pathogenesis of psychoses, are controversial problems. Some authors (McGeer *et al.*, 1956*a*, *b*, 1957; Acheson *et al.*, 1958) regard the reported differences as significant. Others (Armstrong *et al.*, 1958; Shaw and Trevarthen, 1958; Riegelhaupt, 1958; Sano, 1958; Rodnight and Aves, 1958; Curzon, 1958; Cafruni and Domino, 1958; Forrest, 1959; Fleischhacker and Lancaster, 1958, 1959) conclude that the reported qualitative differences have no diagnostic or pathogenetic meaning. They believe that only quantitative evaluation of each compound under strictly controlled dietary conditions could give reliable results.

In the present paper a report is made of research intended to analyse qualitatively and quantitatively the relation between tryptophan degradation and schizophrenia, and to study the metabolism of tryptophan in this disease. The quantitative determination of urinary metabolites excreted by schizophrenic patients after tryptophan administration by pathways leading to the formation of (1) nicotinic acid and through 5-hydroxytryptophan and 5-hydroxytryptamine (serotonin) of (2) 5-hydroxyindoleacetic acid, gives a useful indication of tryptophan metabolism in them and allows comparison with healthy controls. The recent development of a convenient analytical method (Coppini, Benassi and Montorsi, 1959) for urinary tryptophan metabolites has made possible this study in which 8 compounds were determined. This gives a much clearer pattern of tryptophan metabolism than the single determination of xanthurenic acid (Zeller, 1958).

EXPERIMENTAL

The experimental work may be divided into four sections.

(a) Qualitative chromatographic analysis of the urine of 200 chronic schizophrenic patients receiving a standard hospital diet: this was to ascertain the 'spontaneous' elimination of tryptophan metabolites by the kynurenine and serotonin pathways under conditions of standard dietary intake. The overnight urine of the 200 patients and of 30 healthy controls receiving the same diet were analysed by unidimensional descending paper chromatography on Whatman No. 1 paper: the subjects were of different age, sex, and body-weight. Of each urine sample, 0·1 ml was spotted on the paper, without regard to the volume of urine collected: chromatograms were developed using the organic phase of a *n*-butanol–acetic acid–water (4:1:5) mixture. After elimination of the solvent, chromatograms were classified according to the number and intensity of the fluorescent spots which appeared on exposure of the papers to u.v. light of 3655 Å wavelength.

(b) Quantitative determination of tryptophan metabolites of the kynurenine pathway in the urine of 6 healthy controls and of 11 schizophrenic patients after tryptophan loading. Patients ranged from 25 to 40 years and they matched the control subjects in age, weight and sex (all males); all of them had been suffering for more than five years from a chronic dissociation syndrome of a predominantly delirious content with hebephrenic–catatonic manifestations. Three of the control subjects were laboratory and hospital personnel who had no known disease and were fed with a free diet (see Table 1). The other three of the control subjects were patients admitted to the hospital for conditions which seemed unlikely to have metabolic implications. These controls had been on hospital diet for at least 6 months and their mobility was comparable to that of the schizophrenic patients.

Since there seemed to be no indication that the hospitalized patients metabolized tryptophan differently from the subjects with no known disease, the control subjects were placed in one group. L-Tryptophan in doses of 490 μmoles/kg (100 mg/kg body wt) was administered in wafers to the fasted patients and controls early in the morning and the urine was collected in dark bottles under

TABLE 1.—URINARY EXECRETION OF TRYPTOPHAN METABOLITES 24 HR AFTER ADMINISTRATION OF L-TRYPTOPHAN (490 μMOLES/KG) IN SCHIZOPHRENIC PATIENTS AND IN HEALTHY CONTROLS

Case	Administered tryptophan (μmoles)	Kynurenine (μmoles) total	%	N-α-acetylkynurenine (μmoles) total	%	3-Hydroxykynurenine (μmoles) total	%	Kynurenic acid (μmoles) total	%	Xanthurenic acid μmoles total	%	3-Hydroxyanthranilic acid μmoles total	%	Anthranilic acid μmoles total	%	Metabolites sum (μmoles) total	%
SCHIZOPHRENIC PATIENTS																	
A	24500	2346 ± 57*	9·57	300 ± 10	1·23	377 ± 42	1·54	436 ± 13	1·78	146 ± 10	0·62	496 ± 71	2·02	absent		2715	16·74
B	29410	2722 ± 60	9·25	526 ± 25	1·80	216 ± 15	0·74	630 ± 9	1·64	96 ± 3·1	0·34	274 ± 25	0·93	absent		4464	14·70
C	30390	1882 ± 89	6·19	333 ± 39	1·09	320 ± 10	1·05	380 ± 9	1·22	201 ± 3·7	0·66	666 ± 30	2·19	1217 ± 45	4·01	4999	16·41
D	30880	2444 ± 81	7·91	763 ± 49	2·47	328 ± 19	1·06	907 ± 19	2·93	243 ± 12	0·79	65 ± 5	0·21	absent		4750	15·37
E	30880	2922 ± 127	9·46	488 ± 24	1·58	269 ± 23	0·86	867 ± 31	2·80	258 ± 21	0·83	164 ± 6	0·53	absent		4968	16·09
F	31860	3641 ± 32	11·43	386 ± 21	1·21	325 ± 21	1·06	811 ± 24	2·57	119 ± 8·3	0·37	70 ± 4	0·22	absent		5352	16·85
G	36275	2895 ± 20	7·98	734 ± 12	2·02	320 ± 15	1·05	600 ± 30	1·10	274 ± 9·1	0·75	109 ± 8	0·30	absent		4932	13·04
H	37255	5164 ± 10	13·86	597 ± 22	1·60	402 ± 11	1·07	1275 ± 30	3·41	242 ± 16	0·64	960 ± 32	2·57	absent		8640	23·15
I	38740	2866 ± 47	7·65	797 ± 55	2·05	435 ± 36	1·12	1160 ± 29	3·02	151 ± 5·9	0·41	1067 ± 19	2·49	absent		6476	16·74
L	39210	4625 ± 74	11·80	776 ± 29	1·98	207 ± 25	0·53	1607 ± 31	4·10	57 ± 1·5	0·15	278 ± 12	0·71	absent		7550	19·27
M	49000	2817 ± 85	5·75	672 ± 5	1·37	554 ± 24	1·13	479 ± 12	0·98	417 ± 8·3	0·85	830 ± 43	1·70	absent		5769	11·78
Mean		3120 ± 297†	9·17	580 ± 56†	1·68	341 ± 30†	1·02	832 ± 116†	2·33	200 ± 31†	0·58	452 ± 112†	1·26	—		5510 ± 480†	16·05
NORMAL CONTROLS																	
1+	24500	480 ± 62	1·95	157 ± 18	0·64	12 ± 0·25	0·05	445 ± 12	1·81	93 ± 3·2	0·38	280 ± 8	1·14	absent		1467	5·97
2+	27000	575 ± 15	2·13	207 ± 22	0·76	18 ± 0·10	0·06	408 ± 15	1·51	81 ± 5·8	0·30	490 ± 8	1·81	absent		1779	6·57
3°	30882	656 ± 18	2·12	396 ± 13	1·28	52 ± 7·0	0·17	574 ± 5	1·86	76 ± 10·0	0·25	270 ± 17	0·87	absent		2024	6·55
4+	35300	950 ± 55	2·72	358 ± 23	1·01	47 ± 1·5	0·13	697 ± 24	1·97	102 ± 1·7	0·28	705 ± 10	2·61	absent		2859	8·72
5°	38275	1383 ± 41	3·61	193 ± 8	6·50	73 ± 5·1	0·19	315 ± 9	0·82	117 ± 5·6	0·30	665 ± 5	1·73	absent		2746	7·15
6°	39210	1462 ± 98	3·73	158 ± 4	0·40	41 ± 6·8	0·13	374 ± 17	0·96	57 ± 1·9	0·15	275 ± 20	0·71	absent		2367	5·08
Mean		917 ± 172†	2·82	245 ± 43†	0·75	40 ± 9†	0·12	469 ± 58†	1·44	87 ± 9†	0·27	447 ± 82†	1·37	—		2207 ± 224†	6·67
Excretion ratio between schizophrenic patients and healthy controls		3·40		2·36		8·50		1·62		2·29		0·92		—		2·41	

The amounts of metabolites excretes by each of the schizophrenic and healthy subjects are reported both in μmoles and as percent of administered tryptophan.

* ± experimental standard error.

† ± sample standard error. The differences in excretion of schizophrenic and controls were significant at the level $P < 0.005$ for kynurenine, and $P < 0.001$ for acetylkynurenine, kynurenic acid, 3-hydroxykynurenine kynurenine, xanthurenic acid and total metabolites; $P > 0.05$ for 3-hydroxyanthranilic acid,

+ on a free diet.

° on standard hospital diet.

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toluene. The analysis of the urines passed in the 24 hr following the administration was carried out as soon as possible, always within two days; the following substances were determined: kynurenine, N-α-acetylkynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, 3-hydroxyanthranilic acid, anthranilic acid and 5-hydroxyindoleacetic acid. The first seven substances were analysed by the previously described method: 0·05–0·1 ml of urine were spotted on each sheet, the volume depending upon the amount of substances present. Chromatograms were developed according to the bidimensional technique using the above mentioned solvent for the first run and distilled water for the second one (after each run papers were dried in an oven at 40°). After elimination of the solvent, substances were identified, cut out, eluted from the paper and determined spectrophotometrically or colorimetrically.

The urines excreted between the 24th and the 48th hour after administration did not show fluorescent spots and gave the same chromatographic pattern as those collected in the 24 hr preceding administration: the metabolism of administered tryptophan apparently takes place within a day.

(c) Quantitative determination of tryptophan metabolites in the blood of two schizophrenic patients and of one healthy control at different times after the administration of tryptophan. The determinations on blood serum were carried out at 0, 4, 8, 12, 16 and 21 hr after tryptophan loading: 1 ml of serum was deproteinized by mixing with equal volumes of acetone and ethanol. The protein residue was washed several times with the same mixture. The combined extracts were concentrated under vacuum in a N_2 stream, on a water bath at less than 30°, and then submitted to bidimensional paper chromatography. The only metabolite found in serum was kynurenine, which was determined as previously indicated (Coppini *et al.*, 1959).

(d) Quantitative determination of 5-hydroxyindoleacetic acid (the final product of the serotonin pathway) in the urine of 8 chronic schizophrenic patients and of 5 healthy control subjects 24 hr before and 24 hr after tryptophan administration. 5-Hydroxyindoleacetic acid was estimated, in duplicate, using 6 ml of urine according to the method of Udenfriend *et al.* (1955).

RESULTS AND DISCUSSION

'*Spontaneous*' *excretion of tryptophan metabolites*

The chromatograms of the urines of 200 chronic schizophrenics and of 30 controls showed small amounts of xanthurenic acid and, in some cases, of kynurenic acid. Ultraviolet light and spraying either with diazotized sulphanilic acid or with *p*-dimethylaminobenzaldehyde revealed many other spots which did not correspond to any of the metabolites considered in the present investigation.

Urine analysis after tryptophan loading

The quantitative determination of tryptophan metabolites in 11 patients with schizophrenia and in 6 healthy controls after L-tryptophan administration showed that the responses of the two groups were clearly different with respect to the kynurenine pathway. To make the comparison easier, the mean values of the amounts of each metabolite excreted by the schizophrenic and healthy subjects after the test load are given in Table 1. The ratio of the amounts of each substance and of total metabolites excreted by schizophrenics, to the amounts excreted by the controls are also given (Table 1).

Kynurenine is the major metabolite in both groups and represented more than 50 per cent of the total detected tryptophan metabolites excreted by schizophrenic patients. These subjects eliminate in the urine 2·4 times the amount of tryptophan metabolites excreted by normal subjects. The ratio is different for each metabolite: it is highest for 3-hydroxykynurenine, which is eliminated in very small amounts by healthy people (mean value = 0·12 per cent of administered tryptophan) and in amounts about 9 times as large by schizophrenic subjects (mean value = 1·02 per cent of administered tryptophan). It is lowest for 3-hydroxyanthranilic acid, the mean

excretion of which decreases in the pathological condition. The concentration of 3-hydroxyanthranilic acid was low in the urines of three patients with schizophrenia, perhaps because the excretion of 3-hydroxykynurenine, the immediate precursor of 3-hydroxyanthranilic acid, was very high in these subjects; free anthranilic acid was found in the urine of only one schizophrenic patient but in a great amount (4·01 per cent of administered tryptophan). The schizophrenic patients excrete more kynurenic and xanthurenic acids than the healthy controls. The increased excretion is related to the greater amounts of kynurenine and 3-hydroxykynurenine formed by these patients.

The above data emphasize the importance of quantitative determination of more than one urinary metabolite. If xanthurenic acid only had been determined, the abnormality of tryptophan metabolism in schizophrenic patients would have been only partially revealed. The results (Table 1) suggest that transaminase activity is not decreased while kynureninases are inhibited in schizophrenic patients. The abnormality of the tryptophan metabolism of schizophrenic patients suggests a functional pyridoxine deficiency. We have not succeeded in obtaining normal tryptophan metabolism following pyridoxine treatment (100 mg $\times$ day $\times$ 30) in three schizophrenic patients (BENASSI, BENASSI and ALLEGRI, 1960) unlike PRICE, BROWN and PETERS (1959). The effects of vitamins other than pyridoxine on this abnormality is under study.

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Our data do not entirely agree with those recently published by PRICE *et al.* (1959) on tryptophan metabolism in porphyria, schizophrenia and a variety of neurological and psychiatric diseases. These investigators found that 6 patients with schizophrenia had distinctly abnormal tryptophan metabolism and 13 others metabolized the amino acid in a normal manner. The patients with abnormal metabolism excreted significantly more kynurenic acid, N-α-acetylkynurenine, kynurenine, 3-hydroxykynurenine and O-aminohippuric acid after 2·0 g of L-tryptophan (8·72 per cent of dose discharged) than did the controls (2·18 per cent). Further, patients with a variety of other psychoses showed a high incidence (7 out of 8) of abnormal metabolism when given the same amount of tryptophan.

The challenging dose of L-tryptophan used in the present study was about 4 times higher than that of PRICE. Previous experiments by the same investigators (BROWN and PRICE, 1956) showed the total amount of metabolites excreted was about 6·8 per cent in normal subjects after 8·0 g of tryptophan. This value agrees with the average amount excreted (6·77 per cent) by our 6 healthy controls. The schizophrenic patients studied by PRICE *et al.* (1959) included 9 subjects who were experiencing their first psychotic episode, while our patients had all been suffering for more than 5 years from a chronic dissociation syndrome of a predominantly delirious nature and with hebephrenic–catatonic manifestations. In spite of these experimental differences there are many points of agreement between our results and those of PRICE.

Tryptophan metabolites in blood serum

The chromatographic analysis of serum of two schizophrenic and one control subject showed that the only metabolite detected in the samples taken at different times after tryptophan administration was kynurenine. The three curves in Fig. 1 represent the kynurenine levels (μmoles per 100 ml of serum) of two schizophrenic subjects (cases L and B of Table 1) and one healthy control (case 4) after tryptophan administration, as a function of time.

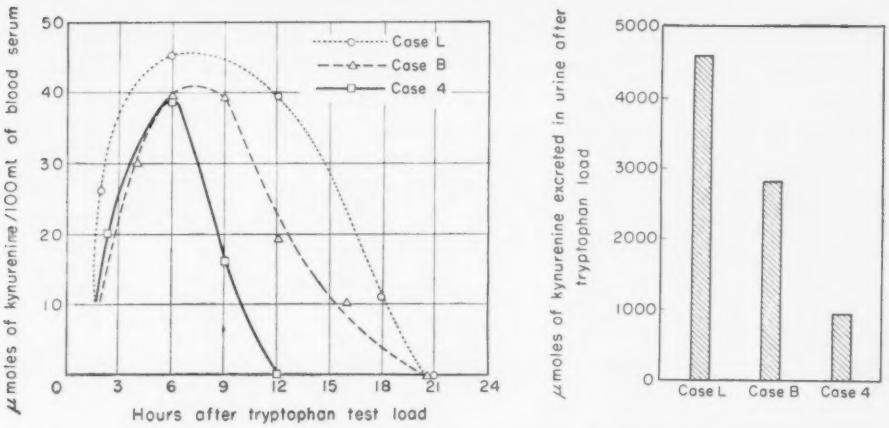

FIG. 1.—Comparison between kynurenine content in μmoles per 100 ml of blood serum at different times and kynurenine in μmoles present in urine collected 24 hr after administration of L-tryptophan to two schizophrenic patients, case L and B, and to one healthy control, case 4 (reported from Table 1).

A comparison of the urinary excretion and of serum levels of kynurenine, shows that the smaller urinary elimination of the compound by normal controls has its counter-part in lower blood levels after tryptophan administration. In a healthy subject (case 4) kynurenine not only reached a lower level in serum than in schizophrenic subjects but disappeared altogether shortly after the twelfth hour.

5-Hydroxyindoleacetic acid determination

The values of excretion of 5-hydroxyindoleacetic acid (Table 2) agree with those of most other authors. Statistical analysis shows that in both groups L-tryptophan administration increased the elimination of 5-hydroxyindoleacetic acid very little, within the limits of daily variations. The difference between the amounts excreted by healthy controls and those, slightly higher, excreted by schizophrenic subjects, is not significant ($P > 0{\cdot}5$); but even in schizophrenic cases the quantities of 5-hydroxyindoleacetic acid metabolized by the serotonin pathway represent only 0·029–0·039 per cent of administered tryptophan.

SUMMARY

Qualitative chromatographic analysis on filter paper of the urines of 200 chronic schizophrenics and of 30 healthy controls gave no evidence of spontaneous excretion of tryptophan metabolites or of a significant difference between the two groups.

TABLE 2. EFFECTS OF L-TRYPTOPHAN ADMINISTRATION (490 μMOLES/KG = 100 MG/KG) ON THE URINARY EXCRETION OF 5-HYDROXYINDOLEACETIC ACID

Cases	No. of subjects	Administered tryptophan (μmoles)*	5-Hydroxyindoleacetic acid (μmoles)*		5-Hydroxyindoleacetic acid	
			24 hr before tryptophan	24 hr after test load	mean increase (μmoles)*	per cent of administered tryptophan
Normal	5	31911	21·41 ± 0·61†	30·50 ± 0·48	9·09 ± 0·25	0·029
Schizophrenic	8	35163	14·74 ± 0·31	26·75 ± 0·56	12·01 ± 0·33	0·039

Difference of mean increase = 2·92 (not significant, $P > 0{\cdot}50$)
* mean values per person
† ± standard error

A quantitative study of excreted metabolites carried out on 11 schizophrenics and on 6 controls after oral administration of tryptophan (490 μmoles/kg = 100 mg/kg) showed that the schizophrenic patients were unable to metabolize normally the administered tryptophan; the metabolites formed by the kynurenine pathway were excreted by the mental patients in amounts 2·4 times greater than by normal controls.

5-Hydroxyindoleacetic acid excretion, which was taken as an index of tryptophan metabolism by the serotonin pathway, was not affected, either in the normal or in the schizophrenic subjects, by the administration of tryptophan.

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A SENSITIVE ASSAY METHOD FOR ADP AND THE DETERMINATION OF ATP, ADP AND CrP IN SINGLE NERVE TRUNKS*

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THE determination of submicromolar amounts of ATP‡ in frog sciatic nerves was first described by GREENGARD, BRINK and COLOWICK (1954). They made use of the luciferase system (MCELROY and STREHLER, 1949) in which, under appropriate conditions, the intensity of a flash was found to be proportional to the amount of ATP present. This paper extends the above method to ADP by coupling in succession several enzyme systems, each followed by a heat-inactivation step, in the following sequences:

(a) a pyrophosphorolysis of ATP in the presence of sulphurylase (ROBBINS and LIPMANN, 1958) and pyrophosphatase (KUNITZ, 1952);

(b) a phosphorylation of ADP to ATP with creatine phosphokinase (NODA, KUBY and LARDY, 1955); and

(c) the final determination of ATP (phosphorylated ADP) with luciferase (STREHLER and MCELROY, 1957).

Choice of an extraction procedure so that a single frog sciatic nerve can be used for the determination of ATP, ADP and CrP is discussed.

MATERIAL AND METHODS

Luciferase was prepared according to the method of MCELROY and COULOMBRE (1952). The first gel supernatant fluid was used as the enzyme preparation. Creatine phosphokinase was prepared according to the procedure of NODA, KUBY and LARDY (1955). The procedure of ROBBINS and LIPMANN (1958) for the purification of sulphurylase was followed. The resin electrophoresis step was required to free the sulphurylase from adenylate kinase. Pyrophosphatase prepared by the method of KUNITZ (1952) was kindly supplied by Dr. KUNITZ of this Institute.

CrP was obtained from the California Corporation for Biochemical Research, and ATP and ADP were purchased from Sigma Chemical Company.

ATP determination

A sensitive photometer assembly designed by Dr. E. F. MACNICHOL, Jr. of the Johns Hopkins University was used to detect the peak light intensity developed in the luciferase system. The signal was fed into a Varian chart-recorder with a one-second full-scale response time. That the recorder did respond fast enough to register faithfully the magnitudes of the flashes was checked in preliminary tests by simultaneous measurements of the photometer signal with an oscilloscope. The light-producing reaction was carried out in a small test tube (10 mm × 75 mm) placed as closely as possible to the photomultiplier within a light-tight box. A hypodermic needle projected through the cover of the box to a point just above the mixture in the test tube so that mixing was rapid when the luciferase solution was injected.

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‡ Abbreviations: AMP, 5′-adenylic acid; ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; Cr, Creatine; CrP, Phosphocreatine; P_I, Inorganic phosphate; PP, Inorganic pyrophosphate; tris, Tris(hydroxymethyl)-amino-methane; PCA, Perchloric acid.

Luciferase activity is affected by high salt concentrations (McELROY, personal communication). The dependence of the peak light intensity emitted by the luciferase system on concentration of tris (buffer) at pH 7·9 is shown in Fig. 1. Final concentrations of 0·05 and 0·10 M were selected for ATP and ADP determinations respectively. Some activity was sacrificed so that the ionic strength might be kept relatively constant. The procedure was carried out as follows: 0·05 ml of M-tris (pH 7·9), 0·30 ml of 10 mM-$MgCl_2$, 0·10 ml of diluted extract (diluted 1:10 with water), and 0·45 ml of H_2O were added to a small test tube and equilibrated at 25°; 0·10 ml of ice-cold solution of luciferase was squirted into the tube immediately after its transfer to the light-tight reaction chamber. The total volume was 1·0 ml. Crude luciferase is rather unstable. A 10 per cent decrease in activity was

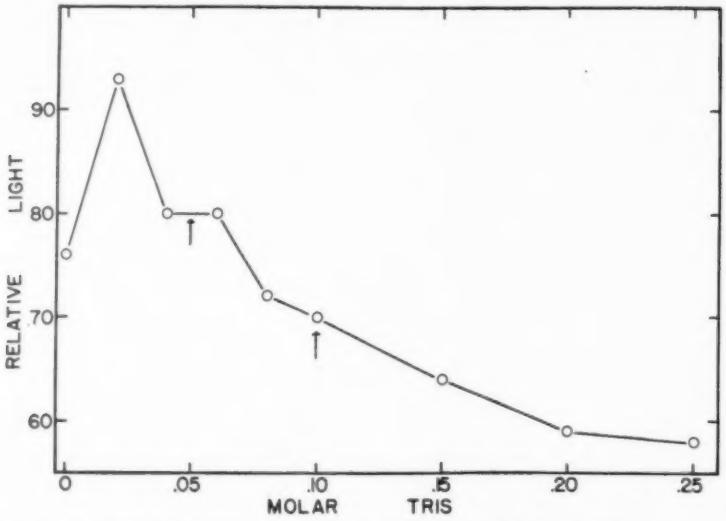

FIG. 1.—Relative flash intensity produced by luciferase as a function of Tris concentration.

sometimes observed after incubation for 30 min at 25°. However, no appreciable loss in activity was found if the enzyme was kept in an ice-bath for the same length of time. Since at least 20–30 min were required for routine measurements, this procedure of double-temperature equilibrium was followed. For each set of determinations several standards were always included. They covered the range of 0–0·10 μm-mole of ATP, and invariably a linear relationship was found between ATP concentration and relative light emission.

The enzymic conversion of ADP to ATP

The ATP present in a portion was first removed by the action of sulphurylase in the presence of molybdate (equation 1).

$$\mathrm{ATP} \xrightarrow[Na_2MoO_4]{\text{sulphurylase}} \mathrm{AMP} + \mathrm{PP} \qquad (1)$$

The inorganic pyrophosphate was destroyed by pyrophosphatase (equation 2).

$$\mathrm{PP} \xrightarrow{\text{pyrophosphatase}} 2\,P_i \qquad (2)$$

When less than 1 μm-mole of ATP was present, the pyrophosphatase reaction was sometimes omitted (McELROY, 1955). Incubation with both enzymes was carried out at pH 6·7. The pH optima for sulphurylase (ROBBINS and LIPMANN, 1958) and pyrophosphatase (KUNITZ, 1952) are, respectively, 7·5–9·0 and 7·0–7·2. After the reactions were completed, these enzymes were inactivated by heat, and the reaction mixture was chilled in an ice bath to minimize the breakdown of ADP. Then CrP and creatine phosphokinase were added to transform ADP to ATP (equation 3).

$$\mathrm{ATP} + \mathrm{Cr} \underset{\text{phosphokinase}}{\overset{\text{creatine}}{\rightleftharpoons}} \mathrm{ADP} + \mathrm{CrP} \qquad (3)$$

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No other compound was added, so the pH remained at 6·7 favouring the backward reaction. The mixture was again heated and chilled. M-Tris was added to raise the pH to 7·9, and the luciferase system was finally added to measure the ATP which had been formed from ADP. The first three enzymes were all free of interfering enzymes, particularly phosphatases and adenylate kinase.

Molybdate was found not to affect the creatine phosphokinase but it did reduce the intensity of the flash from the luciferase system (Fig. 2). However, it is possible to choose a concentration of molybdate which allows a rapid completion of the sulphurylase reaction without causing excessive inhibition of the luciferase system. In Fig. 2, the arrow on the left indicates the K_M value (0·4 mM) for molybdate in the sulphurylase system. The arrow on the right shows the molybdate concentration

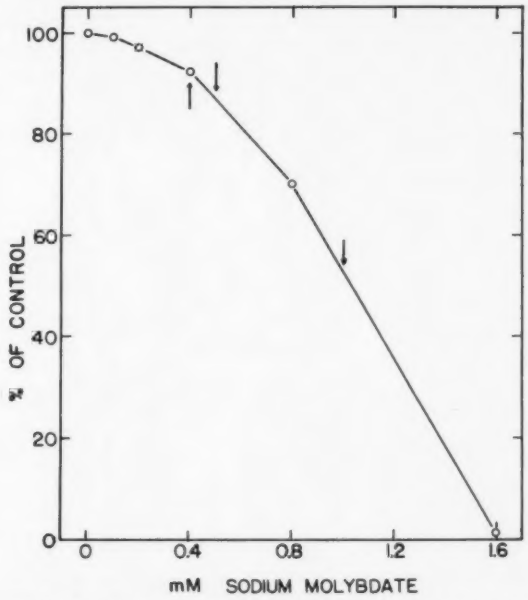

FIG. 2.—Molybdate inhibition of flash intensity by luciferase. See text for explanation

(1·0 mM) during the incubation period with sulphurylase. After the addition of the other components of the assay, molybdate was present at 0·5 mM (middle arrow in Fig. 2). This concentration produced about 10 per cent reduction in peak light intensity. Owing to the inhibition by both molybdate and high buffer concentration, the peak intensity of light generated in the ADP determination was only about a third of that generated by an equal amount of ATP in the ATP determinations. In Fig. 3, curve 1 represents the result of ATP determinations with the method previously described in *ATP determination*, above. Curve 2 was obtained by coupling only the creatine phosphokinase and the luciferase systems. No ATP was added, and the sulphurylase and pyrophosphatase incubation steps were omitted. However, molybdate was included in the reaction mixture. Curve 3 shows the result of ADP determinations with the complete procedure. Each tube in these experiments contained initially 1 μm-mole of ATP in 1 ml which was completely destroyed by pyrophosphorolysis. The ADP determinations were not affected by ATP initially present.

The complete procedure for ADP determination

(a) 0·10 ml maleate buffer (pH 6·7; containing 30 mM-$MgCl_2$ and 50 mM-potassium maleate), 0·10 ml of 5 mM-Na_2MoO_4, 0·20 ml diluted extract (diluted 1:10 with water), 0·02 ml sulphurylase,* 0·01 ml or no pyrophosphatase, and water were added to make a total volume of 0·50 ml in a small test tube. Incubation was at 30° for 25 min.

* The amount of sulphurylase was adjusted so that 1 μm-mole of ATP was completely decomposed in 25 min. The amount of creatine phosphokinase was also adjusted so that 1 μm-mole of ADP was completely phosphorylated to ATP in 25 min.

(b) enzyme inactivation was achieved by immersion for 2 min in a boiling water bath. It was followed by chilling in an ice bath.

(c) 0·03 ml of creatine phosphokinase and 0·10 ml of 0·1 mM-CrP were added, and the sample was reincubated 25 min at 30°.

(d) step (b) was repeated

(e) 0·10 ml of M-tris (pH 7·9) and 0·17 ml H_2O were added, the sample was equilibrated at 25°, and 0·10 ml of luciferase was added.

(f) Standards, containing 0–0·07 μm-mole ADP, were carried through the same procedure for each set of determinations.

CrP determination

The method of ENNOR and STOCKEN (1952) was employed with slight modification. The total

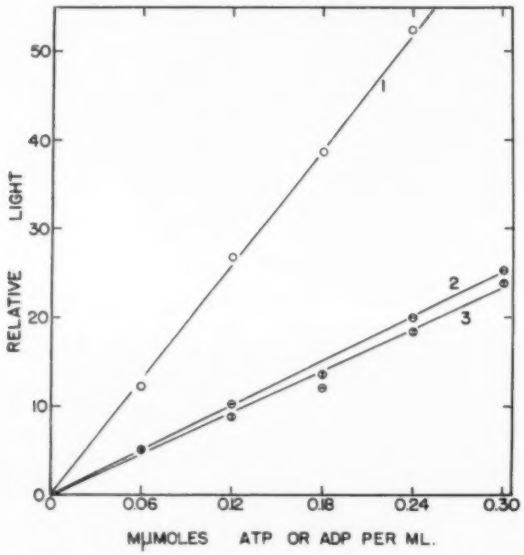

FIG. 3.—Flash intensity by luciferase as a linear function of ATP or ADP concentration. (1) ATP; (2) ADP, no ATP added, sulphurylase and pyrophosphatase omitted; (3) ADP and ATP carried through the entire procedure.

volume was reduced from 10·0 ml to 1·5 ml. The use of *p*-chloromercuribenzoate and the development of colour in the dark were found necessary. The concentrations of α-naphthol and diacetyl were increased to enhance the colour formation.

Two sets of determinations, with and without acid hydrolysis, were carried out. The procedure was:

(a) With hydrolysis: 0·80 ml extract and 0·10 ml H_2O were equilibrated at 65°. 0·10 ml of N-HCl at 65° was added and well mixed. Exactly 9 min later, 0·10 ml of N-NaOH at 0° was added. The tube was immediately shaken and chilled in an ice bath.

(b) Without hydrolysis: As in (a), except all solutions were kept and mixed at ice bath temperatures.

(c) All tubes were returned to room temperature. Then 0·30 ml of fresh α-naphthol solution (0·5 g in 20 ml of stock alkali) (Stock alkali: 60 g NaOH and 160 g Na_2CO_3 per litre solution) and diacetyl (0·4 ml of 1% solution in 25 ml) were added and well mixed. After 25 min the colour that developed was determined at 530 mμ in a 1·0 ml microcuvette with 1·0 cm optical path.

(d) Standards (0–30 μm-moles of creatine) were carried through in each set of determinations.

(e) Calculations: Tube (a) gave the total creatine content in the sample and tube (b) gave the corresponding free creatine content. The difference gave the CrP content in the sample.

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Extraction of phosphorus-containing compounds from frog sciatic nerves

The trichloroacetic acid extraction of CrP used by GERARD and TUPIKOVA (1939) was found to be unsatisfactory; the extract obtained strongly inhibited the luciferase system. GREENGARD *et al.* (1954) used hot Tris to extract ATP. This extract, unfortunately, inhibited the colour formation in the method for creatine determination. The perchloric acid method of ABOOD and GOLDMAN (1956) was followed. This method represented a compromise because the CrP content in the perchloric acid extract was found to be slightly less than that obtained with a trichloroacetic acid extract, and the ATP content somewhat less than that in a tris extract. Without further effort to find a perfect extraction medium, perchloric acid was used for routine work. The following procedure was carried out at ice bath temperature: A frozen frog sciatic nerve (about 20 mg) was homogenized in 0·5 ml of 1·5 M-$HClO_4$. Cold KOH (0·5 ml), after preliminary titration against the 1·5 M-$HClO_4$ to give an end-point just short of neutralization, was added and well mixed. The thick suspension was transferred to a centrifuge tube. Two 0·5 ml portions of water were used to wash the homogenizer, and the washes were added to the suspension. The suspension was neutralized with 0·1 M-KOH (Hydrion paper 6·0–8·0 was used for end-point detection). After centrifugation, the supernatant fluid was transferred to a graduated centrifuge tube. The precipitate was washed by suspension in 0·5 ml of water and recentrifuged, and the wash was added to the extract. The total volume was brought to 4·0 ml with water, and the solution frozen. Samples prepared in this manner could be stored frozen for a week or two without significant change in their phosphate compounds. Just before determination of these compounds, the extract was thawed, recentrifuged, and the precipitate, if any, was discarded. The amount of extract was just enough for duplicate determinations of ATP, ADP, free creatine, and total creatine.

RESULTS AND DISCUSSION

A typical series of analyses demonstrating the adequacy of the above procedures is given in Table 1 which shows the recovery of added ATP, ADP, CrP and Cr in μm-moles. The solution in tube No. 3, the same as that in tube No. 2, was carried through the homogenization procedure as if a nerve had been present. Separate analyses of the two sciatic nerves of a frog (*Rana pipiens*) are shown in tubes Nos. 4*a* and 4*b*. The agreement between paired nerves was usually within 5 per cent.

TABLE 1.—DETERMINATION AND RECOVERY OF PHOSPHATES IN μM-MOLES

Tube no.	Substance added	Substance recovered				
		ATP	ADP	CrP	Cr	CrP + Cr
1*a*	22ATP	19·9	1·7	—	—	—
1*b*	6ADP	0·1	6·0	—	—	—
1*c*	59·5CrP† plus 10·5Cr	—	—	58·4	11·9	70·3
2	T*	21·5	7·6	56·0	14·3	70·3
3	T*	21·9	8·3	54·0	11·9	65·9
4*a*	23·1 mg nerve	14·8	4·4	34·9	12·4	47·3
4*b*	23·0 mg nerve	13·9	4·4	35·6	11·7	47·3
5*a*	21·7 mg nerve	19·5	7·4	46·4	21·1	67·5
5*b*	22·4 mg nerve plus T*	41·5	14·0	102·3	39·5	141·8
5*b* — 5*a*	T*	22·0	6·6	55·9	18·4	74·3

* T = 22ATP + 6ADP + 59·5Cr + 10·5Cr.

All samples were homogenized in PCA except tubes No. 1*a*, 1*b*, 1*c* and 2.

† The sample of CrP added was supposed to contain 70 μm-moles of CrP. Analyses (Expts. 1*c*, 2 and 3) indicated that it contained, on an average, 12·7 μm-moles of free Cr. A different procedure (in UMBREIT, BURRIS, and STAUFFER, 1957) was used to verify this, CrP was found to contain 0·15 mole-fraction as P_i. Thus the 70 μm-mole sample of CrP used contained 10·5 μm-moles of P_i, the same amount of free Cr, and only 59·5 μm-moles of CrP.

To one nerve (No. 5*b*) of a pair, all the compounds above were added, and the difference between this and the control nerve (No. 5*a*) is shown in line 5*b* − 5*a*. The amounts recovered agree satisfactorily with the amounts added.

It should be emphasized that only one four-hundreth of the original nerve sample was taken for ATP analysis. Thus, out of about 20 μm-moles of ATP in a nerve, only 0·05 μm-mole of ATP was actually measured. Similarly, from a nerve containing 6 μm-moles of ADP, the portion used for ADP analysis contained only 0·03 μm-moles of ADP. This sensitivity is also indicated in the finally adopted procedures where the amounts of ATP or ADP in the standards were less than 0·10 or 0·07 μm-moles respectively.

The average concentrations of ATP, ADP, and CrP found in resting frog sciatic

TABLE 2.—CONCENTRATIONS OF ATP, ADP, AND CrP IN RESTING FROG SCIATIC NERVES

Compound	μmoles/g wet nerve ± S.D.	No. of experiments
ATP	0·90 ± 0·14	51
ADP	0·30 ± 0·13	51
CrP	2·02 ± 0·47	51

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nerve (Table 2) are similar to those recorded in the literature. GERARD and TUPIKOVA (1939) found the content of 'pyrophosphate' to be 0·65 and that of CrP to be 2·9, in the same units, with trichloroacetic extraction. GREENGARD, BRINK and COLOWICK (1954), using hot Tris as extractant, found the ATP content to be 1·1–1·9, and ABOOD and GOLDMAN (1956) found the amounts of ATP and CrP with perchloric acid extraction to be 1·25 and 1·5 respectively.

GREENGARD and STRAUB (1959), using entirely different procedures, were able to determine AMP as well as some other phosphates. They found in the rabbit cervical vagus the contents of ATP, ADP, and CrP to be respectively 2·11, 0·28, and 1·96 μmoles per g wet nerve.

Thus, the ADP and CrP values reported in Table 2 are in good agreement with results obtained by other workers. The ATP value is probably slightly lower than it should be because of the compromise involved in the use of perchloric acid as an extractant.

SUMMARY

An enzymic method for the determination of ADP in concentrations below the μm-mole level in an excess of ATP is described.

The choice of a reagent to extract ATP, ADP, and CrP simultaneously is briefly discussed.

In resting frog sciatic nerves, the contents of ATP, ADP, and CrP were found to be 0·90, 0·30, and 2·02 μmoles per g wet nerve, respectively.

Acknowledgements—I wish to express my deepest appreciation to Drs. D. W. BRONK and F. BRINK, Jr. in whose laboratory this work was carried out, and to Dr. C. M. CONNELLY for his unfailing help and advice throughout this work. I am indebted to Dr. P. W. ROBBINS for his advice in the preparation of sulphurylase and to Dr. M. KUNITZ for supplying pyrophosphatase. I also wish to thank Miss ELLEN R. BATT for her technical assistance.

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METABOLISM OF FROG NERVE DURING ACTIVITY AND RECOVERY

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DURING the period 1920–1935, the chemistry of peripheral nerve was studied extensively and much valuable information was obtained (see reviews of GERARD, 1932; HOLMES, 1934, 1935). Workers were hampered then by lack of sensitive techniques and were forced to pool several nerves to obtain significant results. With improved methods, it was found in the late nineteen forties that the resting respiration and the (extra) activity respiration of a single frog sciatic nerve are inhibited differently by different metabolic inhibitors (DOTY and GERARD, 1950; BRINK, BRONK, CARLSON and CONNELLY, 1952). In the case of azide, the resting respiration remains practically uninhibited at concentrations up to 0·2 mM, while the activity respiration is increasingly inhibited. Above 0·2 mM, the respiration is inhibited to the same absolute level whether the nerve is active or not.

Recent developments in methodology now enable the determination of ATP† and ADP in amounts below μm-mole (STREHLER and MCELROY, 1957; CHENG, 1961). In the present paper, the amounts of ATP, ADP, CrP and lactate in single resting sciatic nerves are reported and compared with values given in the literature. Also reported are the changes in the amounts of these compounds in nerve subjected to stimulation, anoxia, or the action of metabolic inhibitors. The participation in the nerve metabolism of two enzyme systems, creatine phosphokinase and adenylate kinase, in interactions among some of these compounds, is discussed. Finally, the (extra) energy dissipation associated with activity in nerve treated with 0·2 mM-azide is estimated and compared with that in uninhibited nerve.

MATERIAL AND METHODS

Both sciatic nerves were removed from each leopard frog (*R. pipiens*)‡ and kept together in a petri dish containing Ringer's solution (A)§. The perineurium was not removed but each nerve was freed of adhering bits of tissue and mounted on a specially made 'T' (Fig. 1, A). In exploratory lactate determinations, nerves were soaked in Ringer's solution (A) for various periods up to 6 hr before they were immersed in hot NaOH. The lactate content decreased during the first hour, and remained more or less constant afterwards. Consequently, nerves were soaked for at least 1 hr in Ringer's solution (A) before they were mounted in individual chambers for experimentation.

The construction of a nerve chamber is shown in Fig. 1, B. The upper stimulating electrode is the anode and supports a nerve on its 'T'. The chamber was filled to the dotted line with 0·3 ml of Ringer's solution (B)‖. A gas mixture of prescribed composition¶ introduced through the side

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† *Abbreviations:* AMP, 5′-adenylic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cr, creatine; CrP, phosphocreatine.

‡ From Hazen, Alburg, Vermont.

§ Ringer's solution (A) used during dissection: 115 mM-NaCl, 2·1 mM-KCl, 1·8 mM-$CaCl_2$.

‖ Ringer's solution (B) used in nerve chamber: 110·6 mM-NaCl, 2·1 mM-KCl, 1·8 mM-$CaCl_2$, 4·8 mM-$NaHCO_3$, 2% CO_2 in gas phase.

¶ Gas mixtures used: (a) 78% N_2, 20% O_2 and 2% CO_2; (b) 98% N_2 and 2% CO_2.

capillary provided gas equilibration and fluid circulation. Stimuli, supramixal for A fibres, were delivered at the rate of 50 per sec for 2 hr when desired.

When lactate was to be determined, each nerve was immersed in 0·1 ml of 0·1 N-NaOH in a test tube heated in a boiling water bath. The solution was chilled and neutralized with an equal volume of 0·1 N-HCl. The Ringer's solution in the nerve chamber, and two distilled water washes of the chamber, were transferred to this tube. The total volume was brought to 2·6 ml, and the solution frozen. The lactate content was determined within 10 days. The colorimetric method of BARKER and

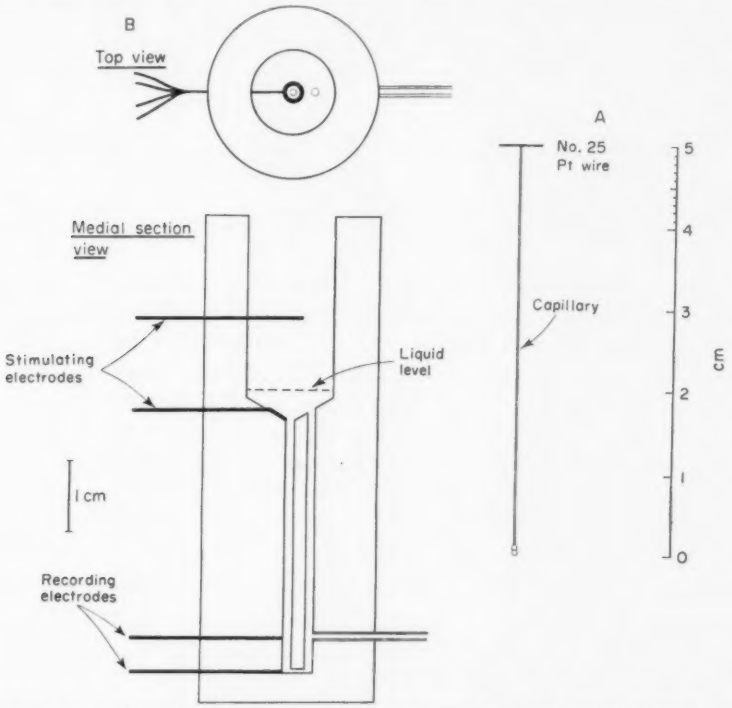

FIG. 1.—Details of experimental chamber and nerve mount ('T'). A: 'T' made of platinum wire and soft-glass capillary. B: Leucite nerve chamber. The upper stimulating electrode (see medial section view) ends in a ring (see top view) which is used to hold the platinum wire of the 'T'.

SUMMERSON (1941) was slightly modified to accommodate samples of this size. Since azide in concentrations up to 0·5 mM reduced colour formation up to 10 per cent, appropriate amounts of azide were included in the standards.

When phosphates were to be determined, nerves were frozen in an acetone–dry ice mixture after each experimental period and were kept frozen until they were homogenized. Homogenization of the nerves and the determination of the phosphates have been described in another paper (CHENG, 1961).

All values are reported as averages ± S.D. in units of μmoles per g wet nerve.

RESULTS

Resting nerves

The average amounts of lactate found in nerves during two separate winter seasons (each season extending from autumn through spring) were both less than 1 μmole per g wet nerve, but differed significantly from each other: 0·98 ± 0·50, $N = 41$ in Table 1 and 0·54 ± 0·16, $N = 20$ in Table 3. The weighted average is 0·83 ± 0·46 with $N = 61$.

TABLE 1.—AMOUNTS OF ATP, ADP, CrP AND LACTATE IN FROG SCIATIC NERVES: EFFECT OF ANOXIA AND/OR STIMULATION

Condition	ATP	ADP	CrP	Σ(~P)*	*N*	Lactate	*N*
Rest	0·90 ± 0·14	0·35 ± 0·09 (0·30 ± 0·13)	1·91 ± 0·39 (2·02 ± 0·47)	4·01 ± 0·59 (4·13 ± 0·62)	51 (39; 44; 32)	0·98 ± 0·50	41
Stimulation	0·81 ± 0·11	0·32 ± 0·11	1·56 ± 0·62	3·58 ± 0·51	45	1·24 ± 0·62	41
Anoxia	0·48 ± 0·12	0·43 ± 0·11	0·70 ± 0·41	2·09 ± 0·57	32	2·71 ± 0·70	14
Anaerobic stimulation	0·35 ± 0·11	0·41 ± 0·09	0·31 ± 0·32	1·42 ± 0·48	43	3·31 ± 0·98	14

* Σ(~P) = 2ATP + ADP + CrP.
All experiments were of 2 hr duration. *N* = No. of analyses.
All values in μmoles per g wet nerve ± S.D.

Average amounts of ADP and CrP in resting nerve, reported previously (CHENG, 1961), are given in parentheses in Table 1. The values not in parentheses in Table 1 are biased averages obtained after exclusion of two groups of observations. Group averages corresponding to separate shipments of frogs occasionally deviated markedly from the over-all (biased) average. Specifically, one shipment yielded an excessively low ADP content (0·12 ± 0·03, $N = 12$), and another (from a Wisconsin breeder) a high CrP content (2·75 ± 0·15, $N = 7$). Statistical analysis, comparing each of these to their respective biased averages, shows $P < 0{\cdot}001$ with $t = 8{\cdot}97$ for ADP and $P < 0{\cdot}001$ with $t = 5{\cdot}57$ for CrP (both with $N = 49$). Σ(~P) signifies the total labile phosphate and is the sum of 2 ATP, 1 ADP and 1 CrP. The biased average for Σ(~P) is obtained after exclusion of the two above mentioned groups of data.

Stimulated nerves

Table 1 also lists the average amounts of ATP, ADP, CrP and lactate found in nerve after 2 hr of activity. This amount of activity had no effect on the content of adenine nucleotides, but caused a 20 per cent decrease in CrP and a similar increase in lactate. Changes, in absolute amounts, can be more clearly demonstrated by experiments in which one nerve of a pair serves as an unstimulated control (Table 2). The results of these experiments are included in the averages in Table 1, but the significance of the difference between the averages is reduced owing to the greater variability between nerves from different frogs than between the nerves of a pair.

TABLE 2.—THE EFFECT OF STIMULATION AND/OR ANOXIA ON THE LEVELS OF ATP, ADP, CrP AND LACTATE; AVERAGE DIFFERENCES BETWEEN NERVE PAIRS ARE TABULATED

	ΔATP	ΔADP	ΔCrP	ΔΣ(~P)	*N*	ΔLactate	*N*
Effect of stimulation: Stimulated − resting	+0·02 ± 0·08	−0·02 ± 0·09	−0·26 ± 0·18	−0·24 ± 0·32	12	+0·40 ± 0·48	10
Effect of stimulation: both nerves anoxic: Stimulated − resting	−0·13 ± 0·07	−0·03 ± 0·09	−0·48 ± 0·48	−0·77 ± 0·46	12	+0·63 ± 0·85	7
Effect of anoxia: Anoxic − resting	−0·43 ± 0·19	+0·13 ± 0·10	−1·64 ± 0·56	−2·38 ± 0·77	6	+2·07 ± 0·43	7

All experiments are of 2 hr duration.
All values in μmoles per g wet nerve ± S.D.
N = number of analyses.

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Anoxia and anaerobic stimulation

Anoxia alone, for 2 hr, produced rather large changes in the amounts of these compounds in nerve (Tables 1 and 2). Table 1 shows that ATP decreased to 53 per cent and CrP to 37 per cent, and that lactate increased to 276 per cent and ADP to 123 per cent. The absolute change in ADP was very small, only 0·08 μmoles per gram in Table 1 and 0·13 in Table 2.

Changes were greater in nerves stimulated during anoxia (2 hr). ATP and CrP were reduced to about 40 per cent and 15 per cent respectively of their levels in resting nerves in oxygen and the lactate increased to 340 per cent. Changes produced by activity during anoxia were similar to those obtained aerobically, except that a decrease in the amount of ATP was also observed. In some nerves, the CrP remaining was negligible. Out of 43 nerve analyses, twelve contained CrP at less than 5 per cent (0·1 μmoles per g) of the level in resting aerobic nerve. This means that CrP as an energy reservoir can be drained almost completely.

Effects of sodium azide and chloretone

In resting nerves, azide caused an increase in lactate, a decrease in CrP and a small decrease in ATP (Fig. 2). With stimulation, nerves in azide contained more lactate, less CrP and less ATP than nerves not in azide. To be specific, 0·2 mM-azide produced in resting nerves about 60 per cent increase in lactate, 50 per cent decrease in CrP and only 10 per cent decrease in ATP. In active nerves the same concentration of azide produced about 100 per cent increase in lactate, 60 per cent decrease in CrP and 20 per cent decrease in ATP, the comparison being made with uninhibited active nerves.

In comparing the effects of stimulation in the presence of azide with those in the absence of azide, the differences in levels of lactate, ATP and CrP in paired nerves (Δ in Fig. 2) illustrate the results more clearly than when over-all averages are compared. The changes, as a result of activity, were larger in the presence than in the absence of azide.

Levels of ADP in both resting and stimulated nerves remained relatively constant.

Results from concurrent paired-nerve experiments, showing the effect of stimulation in anoxic nerves, are included in Fig. 2, on the extreme right of the abscissae scales. A comparison of these with the results obtained with nerves in azide–Ringer's solution, shows that stimulation in 0·2 mM-azide and stimulation in nitrogen produced similar changes in the levels of these compounds.

A few experiments were carried out to compare the effect of the anaesthetic, chloretone, with that of azide. At 2 mM concentration, chloretone reduces the resting respiration of frog nerve to the same degree as does 0·2 mM-azide, namely, to about 80 per cent of its normal value (HURLBUT, 1958). The depression of activity respiration is only one third to one half as great as that produced by 0·2 mM-azide (BRINK, BRONK, CARLSON and CONNELLY, 1952). Table 3 shows that at 1 mM and 2 mM concentrations, chloretone effected no significant change in the amounts of lactate or phosphates in resting nerve, and that it did not modify the changes in these compounds (*i.e.*, the decrease in CrP) produced by activity. The differences between the effects of azide and chloretone on the compounds studied here tend to support the suggestion of HURLBUT (1958) that azide and chloretone may act through different mechanisms.

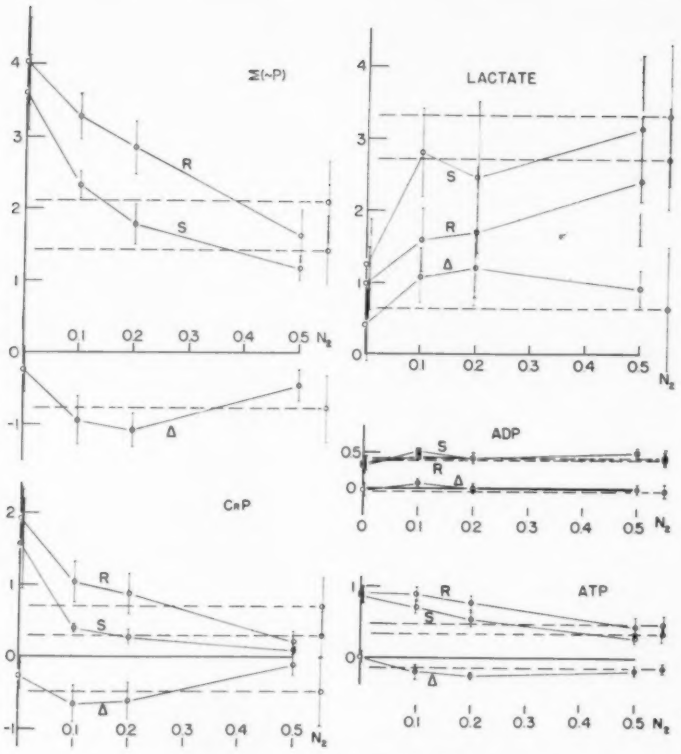

FIG. 2.—Effect of sodium azide on the levels of lactate and phosphates in resting and stimulated nerves.

TABLE 3.—AMOUNTS AND CHANGES OF ATP, ADP, CrP AND LACTATE IN CONTROL AND STIMULATED NERVES. EFFECT OF CHLORETONE

Condition		ATP	ADP	CrP	Σ(~P)	Lactate
Control	R	0·90 ± 0·14(51)	0·35 ± 0·09(39)	1·91 ± 0·39(44)	4·01 ± 0·59(32)	0·54 ± 0·16(20)
	S	0·87 ± 0·11(45)	0·32 ± 0·11(45)	1·56 ± 0·62(45)	3·58 ± 0·51(45)	0·58 ± 0·44(27)
	Δ	+0·02 ± 0·08(12)	−0·02 + 0·09(12)	−0·26 ± 0·18(12)	−0·24 ± 0·32(12)	0·00 ± 0·04(6)
1 mM	R	0·94 ± 0·08(6)	0·40 ± 0·07(6)	1·67 ± 0·14(6)	3·94 ± 0·16(6)	0·62 ± 0·14(14)
	S	0·93 ± 0·06(6)	0·37 ± 0·04(6)	1·43 ± 0·11(6)	3·66 ± 0·15(6)	0·77 ± 0·23(14)
	Δ	−0·01 ± 0·04(6)	−0·03 ± 0·05(6)	−0·24 ± 0·14(6)	−0·28 ± 0·24(6)	+0·11 ± 0·24(7)
2 mM	R	0·87 ± 0·04(6)	0·38 ± 0·05(6)	1·65 ± 0·17(6)	3·77 ± 0·20(6)	0·58 ± 0·14(14)
	S	0·87 ± 0·03(6)	0·38 ± 0·07(6)	1·35 ± 0·25(6)	3·48 ± 0·29(6)	0·70 ± 0·27(21)
	Δ	0·00 ± 0·03(6)	0·00 ± 0·06(6)	−0·28 ± 0·11(6)	−0·29 ± 0·18(6)	+0·06 ± 0·15(7)

All experiments are of 2 hr duration.
All values in μmoles per gram wet nerve ± S.D. No. of determinations in parentheses.
R = Rest. S = Stimulation. Δ = (S − R) paired nerves.

Aerobic recovery after anaerobic stimulation

After a 2 hr period of stimulation in nitrogen, oxygen was readmitted to the chambers and the nerves were allowed to recover in the same bathing solution for 2 hr or 4 hr. During this period the lactate content decreased (Table 4, A) in the presence or absence of 0·2 mM-azide. In the presence of 0·5 mM-azide further increase

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in lactate was observed after 4 hr. During recovery in the absence of azide the amounts of phosphates returned almost to their resting levels after 2–4 hr (Table 4, B).

DISCUSSION

Resting nerves

The weighted mean of 0·83 μmole of lactate per g wet nerve is somewhat less than the 1·2 μmoles per g reported by SAMUELS (1957), but much less than the

TABLE 4.—AEROBIC RECOVERY AFTER ANAEROBIC STIMULATION IN RINGER'S SOLUTION, WITH AND WITHOUT AZIDE

A. Difference in lactate content between recovering and stimulated nerves. (Negative values indicate lactate disappearance.) (Level in stimulated nerve of a pair without rest serves as the baseline.)

	Control ($N = 6$)	0·2mM azide ($N = 6$)	0·5mM azide ($N = 10$)
2 hr rest	$-0{\cdot}26 \pm 0{\cdot}22$, $P < 0{\cdot}05$	$-0{\cdot}27 \pm 0{\cdot}53$, $P < 0{\cdot}4$	$-0{\cdot}19 \pm 0{\cdot}48$, $P < 0{\cdot}3$
4 hr rest	$-0{\cdot}65 \pm 0{\cdot}43$, $P < 0{\cdot}02$	$-0{\cdot}58 \pm 0{\cdot}32$, $P < 0{\cdot}01$	$+0{\cdot}48 \pm 0{\cdot}62$, $P < 0{\cdot}05$

B. Contents of phosphates. (2 and 4 hr rest) ($N = 6$)

ATP	ADP	CrP	$\Sigma(\sim P)$
$0{\cdot}74 \pm 0{\cdot}02$	$0{\cdot}27 \pm 0{\cdot}03$	$2{\cdot}02 \pm 0{\cdot}52$	$3{\cdot}77 \pm 0{\cdot}51$

All nerves were stimulated for 2 hr in nitrogen before aerobic rest.
All values in μmoles per g wet nerve $\pm$ S.D.

TABLE 5.—AMOUNTS OF PHOSPHATES OF SOME PERIPHERAL NERVES

Authors	ATP	ADP	AMP	CrP	Extractant	Source
GERARD and TUPIKOVA	0·65	—	—	2·9	Trichloroacetic acid	Sciatic, green frog
ABOOD and GOLDMAN	1·25	—	—	1·5	Perchloric acid	Sciatic, bull frog
GREENGARD, BRINK, and COLOWICK	1·1–1·9	—	—	—	Hot tris-(hydroxy-methyl)-amino-methane	Sciatic, leopard frog
GREENGARD and STRAUB	2·11	0·28	0·08	1·96	Hot triethanolamine	Cervical vagus, rabbit
CHENG (this paper)	0·90	0·35	—	1·91	Perchloric acid	Sciatic, leopard frog

All values in μmoles per g wet nerve.

3·7 μmoles per g reported by SCHMIDT and CORI (1933). The difference might be due to the fact that SCHMIDT and CORI analysed nerves immediately after dissection without preliminary soaking in Ringer's solution. In our results from two separate seasons a significant difference between the averages was observed. This might have resulted from different nutritional states of the frogs since the handling of the nerves and the assay method remained unchanged.

In Table 5, the present results (biased averages) are shown with those previously

reported in the literature. The values for ATP and CrP presented here are in general agreement with those for ATP and CrP for frog nerves reported by other workers. The recent observations by GREENGARD and STRAUB (1959) on rabbit nerve are included in Table 5 as an example of a similar analysis of a different kind of nerve.

Effect of stimulation

Although the results of SCHMIDT and CORI (1933) agreed with those of earlier reports that the lactic acid content of nerves was not increased by stimulation, SAMUELS (1957) did observe a significant increase in lactate. Data presented here (Table 1) confirm that there is an increase, although it is less than that reported by SAMUELS. He used a higher frequency, 100 per sec, in stimulation and this may account for the difference.

GERARD and TUPIKOVA (1939) reported no change with stimulation in the amount of 'pyrophosphate' in green frog sciatics and a decrease in bull-frog sciatics. ABOOD and GOLDMAN (1956) reported an increase in ATP in bull-frog sciatics when stimulated. In all these, the reported changes were small. The present findings fail to demonstrate any definite change due to stimulation in the ATP content of leopard frog sciatics. GREENGARD and STRAUB (1959) recently demonstrated a definite decrease in ATP in rabbit C fibres in which the ionic exchange due to activity and the associated metabolism may be presumed to be much larger than in frog A fibres. It is clear that if any change in ATP does take place in these A fibres as a result of activity, it will be difficult to determine with certainty.

The finding of a decrease in CrP with stimulation agrees with earlier results (GERARD and TUPIKOVA, 1938; ABOOD and GOLDMAN, 1956). This is consistent with the belief that CrP serves as an energy reservoir, and that it replenishes ATP through the creatine phosphokinase system. In general, stimulation for 2 hr at a frequency of 50 per sec seems not be be a severe treatment for the frog sciatics. This amount of activity did not greatly reduce the action potential, nor did it seriously deplete the known energy reserves.

Effects of anoxia and anaerobic stimulation

During anoxia, both ATP and CrP decrease and lactate increases, whereas ADP increases very little. Stimulation of anoxic nerve does not produce a significant increase in lactate but does cause a definite decrease in ATP; CrP decreases in some cases to zero. The latter finding is in agreement with the earlier work of GERARD and TUPIKOVA (1939); however, their finding of no change in 'pyrophosphate' is not confirmed by the ATP analysis.

Anaerobic stimulation represents the most severe condition used in these experiments. Uniformly, within the 2 hr period, nerve conduction failed. However, the complete utilization (or disappearance) of CrP almost certainly was not the result of irreversible deterioration of the nerve, for restoration of ATP and CrP to normal levels was largely complete after 2–4 hr of recovery in oxygenated Ringer's solution (Table 4, B).

Adenine nucleotide balance and the adenylate kinase system

Since changes in ADP remained small in comparison with changes in ATP under all imposed conditions, a one-to-one correspondence of ATP decrease and ADP

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increase does not exist. The net decrease in the sum of these two adenine nucleotides might be found as AMP. Unfortunately, we were not able to determine AMP at the μm-mole level. An attempt was made to understand why the ADP concentration remained relatively constant. It might be expected that the utilization of ATP by any energy-requiring process would be partially offset by the regeneration of ATP through the adenylate kinase reaction:—

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}, \qquad \text{and} \qquad K_A = (\text{ADP})^2/(\text{ATP})(\text{AMP}).$$

If it is assumed (i) that adenine nucleotides are conserved, and (ii) that adenylate kinase maintains an equilibrium state, then the quadratic term in the numerator of the expression for K_A indicates that changes in ADP will be small in comparison with changes in ATP and AMP. By assuming the value for AMP concentration in resting nerve to be 0·1 μmole per g, based on the results of GREENGARD and STRAUB (1959), we can estimate the value of K_A to be 1·36. Furthermore, we can calculate that the ADP concentration should have varied only between 0·35 and 0·5 μmole per g when the ATP concentration varied between 0·1 and 0·9 μmole per g. This calculated ADP concentration range coincides with the range of observed values. The equilibrium constant (K_A) for rabbit muscle adenylate kinase *in vitro* was found to be approximately unity (KALCKAR, 1943; NODA, 1958). The mean K_A values for nerves under various conditions were calculated to range from 0·74 to 1·36. This agreement strengthens the assumptions made of the concentration of AMP, of the conservation of the adenine nucleotides, and of the maintenance of an equilibrium state by the adenylate kinase.

The creatine phosphokinase system

The concentrations of ATP, ADP, Cr and CrP obtained from each nerve were used to calculate the ratio, $K_C = (\text{ADP})(\text{CrP})/(\text{ATP})(\text{Cr})$, which, *in vitro*, would represent the equilibrium constant of the creatine phosphokinase reaction. The values of K_C for resting and stimulated nerves were 0·44 and 0·29, respectively; those under anaerobic conditions were 0·18 and 0·10, respectively. Clearly, an equilibrium state was not maintained under various conditions. The value of 0·44, for resting nerve, is much higher than the *in vitro* values of 0·01–0·25 reported by NODA, KUBY and LARDY (1954) or the *in vivo* value, 0·05, estimated by CARLSON and SIGER (1959). Suggestions were made (CHANCE and CONNELLY, 1957; CARLSON and SIGER, 1959) that the metabolically active ADP (*i.e.*, the effective ADP concentration) is much less than that found by chemical analysis (MUNCH-PETERSON, 1953; FLECKENSTEIN *et al.*, 1954). The assumption of an effectively low ADP concentration would bring the value of K_C for resting nerve into the range reported by NODA, KUBY and LARDY (1954), but without further assumptions would be incompatible with the maintenance of equilibrium by the adenylate kinase. The question of equilibrium is thus unresolved.

Energetics of nerve metabolism associated with activity

Recently, BRINK (1957), HODGKIN (1957) and CONNELLY (1959) discussed the possibility that the 'high-energy phosphate bond', or ~P, in ATP is the source of energy for maintaining nerve function. Direct evidence supporting this suggestion is

the restoration of Na^+ efflux by ATP injected into poisoned squid axons (CALDWELL and KEYNES, 1957; CALDWELL, HODGKIN and SHAW, 1959). The present investigation complements earlier studies of nerve metabolism (BRINK, BRONK, CARLSON and CONNELLY, 1952; ASANO and HURLBUT, 1958) and makes it possible now to estimate the total energy consumption associated with activity, both in normal and in azide-treated nerves. All energy terms are expressed as equivalent changes in ~P (μmoles per g wet nerve) taking place during 2 hr of impulse conduction at a frequency of 50 per sec.

The increase in respiration during activity was 1·34 μmoles of oxygen per g of

TABLE 6.—VARIOUS FORMS AND AMOUNTS OF ENERGY ASSOCIATED WITH NERVE ACTIVITY*

Environment	Energy consumption				Energy debt	Total††
	Respiration†	Lactate‡	Σ(~P)§	Subtotal‖	Sodium**	
Normal	8·0	0·60	0·24	8·84	9·95	18·79
0·2 mM-azide	0	1·80	1·07	2·87	16·1	18·97

* All values are expressed in μmoles of ~P (or Na^+) per g wet nerve for a 2 hr experiment. They are the differences between values for stimulated and resting nerves. See text for full discussion.

† in μmoles of ~P, converted from oxygen value in Table 3 of BRINK, BRONK, CARLSON and CONNELLY (1952).

‡ in μmoles of ~P, converted from lactate values in Table 2.

§ in μmoles of ~P, (Table 2).

‖ sum of respiration, lactate, and Σ(~P).

** in μmoles of sodium ion from Table 1 of ASANO and HURLBUT (1958).

†† sum of subtotal and sodium, it being assumed, (see Discussion) that the extrusion of one sodium requires one ~P.

nerve (BRINK, BRONK, CARLSON and CONNELLY, 1952). This is equivalent to the production of 8·0 μmoles of ~P (Table 6) if a conversion factor of P:O = 3 is used. The increase in lactate was 0·40 μmole per g (Table 2), equivalent to 0·60 μmole of ~P, if for the formation of every two molecules of lactate three of ADP were phosphorylated to ATP (via glycolysis of polysaccharide). It is assumed that the ~P generated by respiration and glycolysis was consumed during nerve activity. In addition, the decrease in Σ(~P) was 0·24 μmole (Tables 2 and 6). The apparent consumption for 2 hr of activity was thus 8·84 μmoles of ~P per g (Subtotal in Table 6). However, at the end of the activity, there was an additional energy deficit, measured by the accumulation of sodium ions inside the axons, amounting to 9·95 μmoles of Na^+ per g (ASANO and HURLBUT, 1958). There are reasons for assuming that the extrusion of one sodium ion from frog nerve utilizes one ~P (CONNELLY, 1959). Therefore, the accumulated sodium represented an energy deficit of 9·95 μmoles of ~P. The total energy dissipation associated with 2 hr of activity was thus 18·79 μmoles of ~P per g (Total in Table 6) or 52·5 $\mu\mu$moles of ~P per impulse per g.

In nerves treated with 0·2 mM-azide, there was negligible increase in oxygen uptake during activity. The values of other energy terms were all changed: 1·80 μmoles for glycolysis, 1·07 for Σ(~P), and 16·1 for Na^+. However, the estimated total, 18·97 μmoles per g, is commensurate with that given for unpoisoned nerve. This

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approximate energy balance under altered conditions further substantiates the above analysis of our accumulated data and suggests that all major energy changes have been measured.

SUMMARY

The amounts of lactate and phosphates found in resting frog nerve were comparable to results obtained by other workers. The values for lactate, ATP, ADP and CrP in μmoles per g wet nerve are 0·83, 0·90, 0·35 and 1·91, respectively.

Stimulation of frog sciatic nerves at a frequency of 50 per sec for 2 hr was found to be a rather moderate treatment. Only the amount of CrP decreased slightly and the rate of lactate production increased slightly.

Larger changes occurred as a result of anoxia. Results reported in the literature were substantiated.

The ADP concentration in most nerves under various experimental conditions was relatively constant. The adenylate kinase and the creatine phosphokinase reactions are discussed.

When nerve activity respiration was suppressed by appropriate concentrations of azide or chloretone, azide increased glycolysis and lowered the ATP and CrP concentrations, but chloretone had no effect.

The energy requirement for nerve activity is discussed.

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THE HIGH-ENERGY PHOSPHATES IN DEVELOPING BRAIN*

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FOR SOME time investigators have studied the changes in energy metabolism which occur during development of the brain. A rapid rise in the rate of cerebral oxygen consumption has been demonstrated (TYLER, 1942; HIMWICH, 1941*a*, *b*) and the susceptibility of the brain to oxygen deprivation has also been shown to increase during this time. The implication is that the cerebral power (energy per unit time) requirement increases with the maturation of the brain (SAMSON, 1957, 1958). The steady state concentration of ATP† is about the same throughout the maturation period (SAMSON, 1960). The triphosphate compounds of guanosine, cytidine and uridine are now known to be present in brain (SCHMITZ, 1954; THOMAS, 1957; GERLACH, 1958). Indeed, the work of HEALD (1957*b*) established that the GTP concentration in the brain is about 20 per cent of the ATP concentration. However, the other compounds have not been extensively studied. It seemed interesting, therefore, to determine the pattern of these high-energy-phosphate compounds, including phosphoryl-creatine, during neonatal maturation. With this in mind, the following experiments were carried out on rats in different stages of development.

METHOD

Preparation of acid-soluble fraction. Albino rats of accurately known ages (1-day, 5-day, 10-day, or 21-day) from a Sprague-Dawley strain were employed for these experiments. The rats were rapidly frozen by dropping the entire animal into liquid nitrogen. Brain samples were removed either by making slices of the head with a saw, previously cooled with liquid N_2, and then removing the skull and pia mater covering by chipping with a small pick and an 18 gauge hypodermic needle, or by removing the skull and brain with an 18 gauge needle alone. Caution was taken to keep the sample very cold throughout the entire operation. The frozen brain samples were rapidly weighed on a Roller-Smith balance in small copper mesh baskets cooled to the temperature of liquid N_2. Water condensation on the tissue does not seem to be a serious problem if the tissue is removed and weighed quickly. Indeed, a determination of the per cent dry weight of frozen and fresh tissue showed the water content of frozen tissue to vary by less than 1 per cent from that of fresh tissue. The condensation

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† Abbreviations used in the paper are as follows: PCA: perchloric acid; ATP, ADP, and AMP: adenosine tri-, di-, and monophosphates; GTP, GDP, and GMP: guanosine tri-, di-, and monophosphate; UTP, UDP, and UMP: uridine tri-, di-, and monophosphate; UDPAG: uridine diphosphate acetyl-glucosamine; UDPG: uridine diphosphate glucose; CTP, CDP, CMP: cytidine tri-, di-, and monophosphate; NAD and NADH: nicotinamideadenine dinucleotide (oxygenated and reduced); NADP: nicotinamide-adenine dinucleotide phosphate; PCr: phosphoryl-creatine;

$$\text{C.I.: (Confidence Interval)} = \bar{X} \pm \frac{t_{0.95}S}{\sqrt{n}}$$

where

$$\bar{X} = \frac{\Sigma X}{n}$$

X = any experimental value,

and

$$S = \sqrt{\frac{\Sigma X^2 - (\Sigma X)^2/n}{(n-1)}}$$

on the small basket was checked by weighing the basket before and after each tissue measurement. Samples of tissue 150–200 mg in weight were homogenized in ice-cold 20% perchloric acid (1 ml/100 mg tissue) with a Teflon pestle in a glass homogenizer. The tissue was homogenized for 4 min, and the volume adjusted to 10 ml with ice-cold distilled water. The extracts of 1·0–1·5 g brain were pooled. From this pooled extract an amount equivalent to 800 mg tissue was taken for the ion-exchange procedure and the remainder was used for the inorganic phosphate and phosphoryl-creatine analyses.

Preparation of ion-exchange column. The resin was obtained as Dowex (chloride) × 8, 200–400 mesh, and was converted to the formate form as suggested by HURLBERT (1954). It was poured as a thin slurry into a standard chromatography column equipped with a sintered glass plate and adapted for an air tight system. The amount used made a column of 0·75 × 10 cm. The resin was washed only with distilled water to neutrality in most determinations. Washing the column with 6 N-formic acid and 1 M-sodium formate followed by several bed volumes of 88% formic acid, as recommended by some authors, was omitted after the first few experiments, because this washing did not alter the performance of our resin columns or reduce the background ultraviolet light absorption.

Nucleotide analysis. The brain extract, prepared as described above, was adjusted to pH 6–7 with 5 N-KOH and centrifuged at 12,100 **g** for 30–60 min in a Servall model SS-1 angle head refrigerated centrifuge. The temperature was maintained as close to 0° as possible without freezing. The entire supernatant fluid was put on the chromatography resin under pressure at the rate of 1·5–2 ml/min, and the resin was rinsed with 50–100 ml of distilled water to remove the loosely bound or non-bound compounds. The compounds were eluted from the resin by gradient elution techniques as described by HURLBERT (1954). The mixing flask contained 500 ml of liquid throughout the elution and was not changed at any time during the run. The reservoir concentrations used and the positions at which they were changed were as follows: 4·0 N-formic acid from tubes 1 to 18; 8·5 N-formic acid from tubes 19 to 63; 4·0 N-formic acid plus 0·5 M-ammonium formate from tubes 64 to 90–95; 4·0 N-formic acid plus 1·0 M-ammonium formate from tubes 91–96 to 110–115; 4·0 N-formic acid plus 2·0 M-ammonium formate from tubes 111–116 to conclusion.

The eluate was collected in 5·4 ml fractions by means of an automatic fractionator. The optical density of the effluent was continuously monitored at 265 mμ. The optical density peaks were identified by the 280 mμ/260 mμ ratio and by comparison with chromatograms reported in the literature. Determinations with known compounds confirmed the elution characteristics that are reported for this procedure. After appropriate correction for the baseline absorption of the eluent, the quantitative measure of the nucleotides was calculated from standard curves prepared on each of the nucleotides. Some contamination of the ATP peak with UDP and of the GTP peak with UTP was found. These compounds were further separated by rechromatographing the peaks involved after lyophylization. The material remaining after 10–12 hr of lyophylization was diluted to 100 ml with distilled water and put on a resin column prepared in the same manner as described above. Separate runs were made for ATP + UDP and GTP + UTP. The elution procedure for ATP + UDP was 2·0 M-ammonium formate in the reservoir from tube 1 to the conclusion. The procedure for GTP + UTP was: 4·0 M-ammonium formate in the reservoir from tube 1 to 50 and 2·0 M-ammonium formate plus 4·0 N-formic acid from tube 51 to the conclusion. In both cases the mixing flask contained 250 ml. The optical density peaks were quantitatively measured as described above.

Inorganic phosphate and phosphoryl-creatine analyses. The portion taken from the brain extract for inorganic phosphate and phosphoryl-creatine analyses was centrifuged at 7710 **g** in a Servall model SS-1 angle head refrigerated centrifuge for 15–20 min to deposit the PCA insoluble material. The procedure for separating phosphoryl-creatine and inorganic phosphorus was similar to that originally reported by KERR (1935) and STONE (1940) modified by MCILWAIN (1951). Twenty-five ml of the above supernatant fluid containing 1 drop of 0·1% phenolphthalein were placed in a cold homogenization tube, and while homogenizing the pH was adjusted to 8·2 by the introduction of solid $Ca(OH)_2$. The suspension was transferred to a cold graduated cylinder and the tube rinsed with 4 ml of absolute alcohol. The rinsing alcohol was added to the suspension, the volume adjusted to 30·0 ml with distilled H_2O, and the sample allowed to stand 15 min at 0°. It was centrifuged at 12,100 **g** for 20–25 min and the supernatant fluid was analysed for total creatine and free creatine by the method of ENNOR and ROSENBERG (1952). The precipitate was re-dissolved in 0·5 ml of 2 N-HCl and diluted to 10 or 20 ml, depending upon the amount of precipitate, and the inorganic phosphorus was determined by the method of MARSH (1959).

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RESULTS

A typical optical density profile (Fig. 1) shows that the separation of the nucleotides is reasonably clear cut. The tubes that appeared to contain mixtures were lyopholized and rechromatographed to get better resolution. In a number of cases the ATP was independently determined by the firefly luminescence method and the

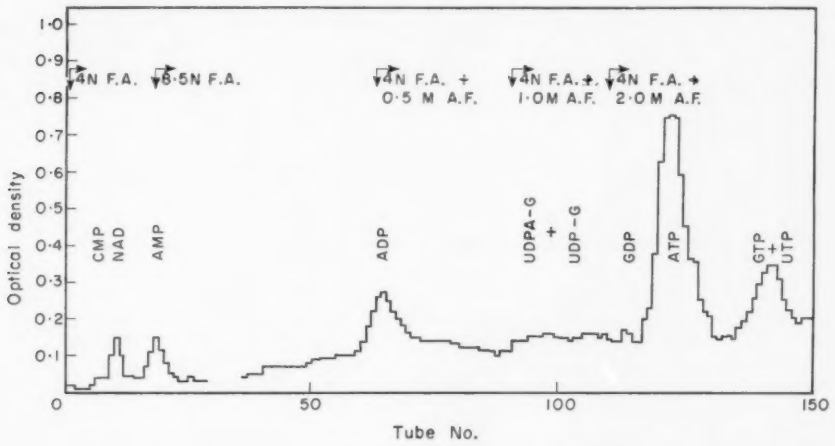

FIG. 1.—Typical chromatogram of the acid-soluble fraction from 3 pooled, 10-day rat brains of total weight 800 mg. Column 0·75 × 10 cm, Dowex 1 (formate); mixer, 500 ml; reservoir contained formic acid (F.A.) alone or with ammonium formate (A.F.) as indicated. Each step in the chromatogram represents a 5·4 ml sample eluted from the column.

values were found to be substantially the same as those from the ion-exchange procedures.

Adenosine phosphates. There was a small but significant increase in the total sum of the adenosine compounds with increasing age (Table 1). The ADP changed more than either the ATP or AMP. The ratio of ATP/ADP, therefore, decreased with maturation.

Guanosine phosphates. As reported earlier by other workers (HEALD, 1957*b*; GERLACH, 1958) there is a considerable amount of GTP in brain (Table 2). In our experiments there was more variation in the GTP than in the ATP concentration. A higher mean concentration of GTP was found in the 5-day brain than in the other ages. The GDP and GMP concentrations appeared to be very low.

Uridine phosphates. UTP was present in appreciable quantities with an almost equal amount of uridine diphosphate compounds (Table 3). UMP was detected in brain samples from all ages but was present in very small quantities.

Cytidine phosphates. It is interesting that the monophosphate was always present in quantities of about 0·018 μmole per g of tissue (Table 4) but the tri- and diphosphates were always below a detectable amount.

Nicotinamide-adenine dinucleotide. As reported by GLOCK and MCLEAN (1955) NAD was present in brain in far larger quantities than was NADH. In our studies NADH was not detected. The data in Table 5 suggest that the NAD concentration may be higher at 5 and 21 days than at 1 and 10 days.

TABLE 1.—ADENINE NUCLEOTIDE CONTENT OF RAT BRAIN

	(μMoles per g of frozen brain)			
	Age in days			
	1	5	10	21
ATP	2·02	2·13	2·27	2·09
	2·02	2·53	2·43	1·98
	1·94	1·77	1·94	2·07
	1·89	1·98	1·74	2·06
	—	—	—	1·77
Mean ± 95% C.I.	1·97 ± 0·10	2·10 ± 0·51	2·10 ± 0·50	1·99 ± 0·16
ADP	0·10	0·11	0·20	0·57
	0·15	0·12	0·22	0·31
	0·14	0·30	0·37	0·31
	0·15	0·25	0·56	0·48
	—	—	—	0·30
Mean ± 95% C.I.	0·14 ± 0·04	0·20 ± 0·15	0·34 ± 0·26	0·39 ± 0·05
AMP	0·12	0·13	0·25	0·19
	0·18	0·17	0·10	0·23
	0·10	0·14	0·12	0·14
	0·05	0·10	0·19	0·17
	—	—	—	0·14
Mean ± 95% C.I.	0·11 ± 0·08	0·14 ± 0·05	0·16 ± 0·11	0·17 ± 0·05
Σ Adenine nucleotide	2·24	2·37	2·52	2·85
	2·35	2·82	2·75	2·52
	2·18	2·21	2·43	2·52
	2·09	2·33	2·49	2·71
	—	—	—	2·21
Mean ± 95% C.I.	2·22 ± 0·17	2·43 ± 0·42	2·55 ± 0·22	2·56 ± 0·30
ATP/ADP	20·2	19·4	11·4	3·7
	13·5	21·0	11·0	6·4
	13·8	5·9	5·2	6·7
	12·6	7·9	3·1	4·3
	—	—	—	5·9
Mean	15·02	13·55	7·68	5·4

Each value was obtained from pooled brain acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10-day, 3; 21-day, 2. Within each age group, for all tables, the nucleotide values for each experimental run lie in corresponding positions.

Creatine and phosphoryl-creatine. The creatine and phosphoryl-creatine concentrations in brain have been measured frequently. However, this appears to be the first time that the total creatine in the brain has been shown to change with maturation (Table 6). The phosphoryl-creatine is considerably lower in the 21-day rats and, therefore, there is an increase in the creatine/PCr ratio from 1·0 to 3·4. This increase, however, may be the result of the slower rate of freezing in the older rats.

Inorganic phosphate. The methods used did not give reproducible results for phosphate in any one age group. The method of MARSH (1959) which prevents the hydrolysis of the ATP by the acid molybdate was used and the values were checked

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TABLE 2.—GUANINE NUCLEOTIDE CONTENT OF RAT BRAIN

	(μMoles per g of frozen brain)			
	Age in days			
	1	5	10	21
GTP	0·58 0·59 0·32 1·26	1·59 1·31 0·73 0·79	0·75 0·77 1·00 0·44	0·55 0·51 0·84 0·73
Mean ± 95% C.I.	0·69 ± 0·64	1·10 ± 0·66	0·74 ± 0·50	0·66 ± 0·25
GDP	<0·01 <0·01 <0·01 0·02	<0·01 <0·01 <0·01 0·04	<0·01 <0·01 0·03 0·04	<0·01
GMP	0·07 <0·01 <0·01 <0·01	<0·01	<0·01	<0·01
Σ Guanine nucleotide	0·65 0·59 0·32 1·28	1·59 1·31 0·73 0·83	0·75 0·77 1·03 0·48	0·55 0·51 0·84 0·73
Mean ± 95% C.I.	0·71 ± 0·65	1·11 ± 0·65	0·76 ± 0·36	0·66 ± 0·25

Each value was obtained from pooled brain acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10-day, 3; 21-day, 2. Within each age group, for all tables, the nucleotide values for each experimental run lie in corresponding positions.

by the GOMORI (1941) modification of the FISKE and SUBBAROW (1925) procedure, but a wide range of values was obtained with both methods. We have recently used an ion exchange procedure for inorganic phosphate which seems to give reproducible results. As yet we do not have a sufficient number of observations to justify a report.

DISCUSSION

It is generally accepted that essentially all the energy used in the activities of the cell will at some point flow through the so-called high-energy phosphates. In view of the 'power-requirement' changes with the maturation of the neonatal brain, it is striking to see that the steady state levels of the high-energy phosphates at different ages are little changed. If the total high-energy phosphates are considered, *i.e.*, two high-energy phosphates from the nucleotide triphosphates, one from the nucleotide diphosphates, and one from phosphoryl-creatine, the total does not change markedly during neonatal maturation (9·44 μmoles/g, 1-day; 10·28 μmoles/g, 5-days; 9·61 μmoles/g,10-days; 8·78 μmoles/g, 21-days). A related compound which does change is the total creatine, which increases significantly with maturation. It is observed that the change is entirely in the free creatine and that the phosphoryl-creatine actually decreases, although the great lability of phosphoryl-creatine makes its determination less reliable than that of the total creatine.

TABLE 3.—URIDINE NUCLEOTIDE CONTENT OF RAT BRAIN

	(μmole per g of frozen brain)			
	Age in days			
	1	5	10	21
UTP	0·27	—	0·20	0·29
	0·21	—	0·23	—
	0·16	0·39	0·17	0·41
	0·64	0·47	0·24	0·39
Mean ± 95% C.I.	0·32 ± 0·35	0·43 ± 0·51	0·21 ± 0·05	0·36 ± 0·16
UDP	<0·01	<0·01	<0·01	<0·01
UDP-Acetyl-glucosamine + UDP-Glucose	0·42	0·22	0·26	0·21
	0·34	0·19	0·47	0·23
	0·36	0·13	0·25	0·19
	0·29	0·21	0·33	0·13
Mean ± 95% C.I.	0·35 ± 0·08	0·19 ± 0·06	0·33 ± 0·02	0·19 ± 0·07
UMP	0·04		0·04	<0·01
	<0·01		0·05	0·05
	<0·01	<0·01	<0·01	0·12
	<0·01		<0·01	<0·01
Σ Uridine nucleotide	0·73	—	0·50	0·50
	0·55	—	0·75	—
	0·52	0·52	0·42	0·72
	0·93	0·68	0·57	0·52
Mean ± 95% C.I.	0·68 ± 0·86	0·60 ± 1·0	0·56 ± 0·22	0·58 ± 0·30

Each value was obtained from pooled brain acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10-day, 3; 21-day, 2. Within each age group, for all tables, the nucleotide values for each experimental run lie in corresponding positions.

TABLE 4.—CYTIDINE NUCLEOTIDE CONTENT OF RAT BRAIN

	(μmole per g of frozen brain)			
	Age in days			
	1	5	10	21
CTP	<0·01	<0·01	<0·01	<0·01
CDP	<0·01	<0·01	<0·01	<0·01
CMP	0·10	—	0·19	0·10
	0·14	0·16	—	—
	0·24	0·14	0·30	0·16
	0·22	0·22	0·27	0·14
Mean	0·17	0·17	0·25	0·13

Each value was obtained from pooled brain acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10 day, 3; 21-day, 2. Within each age group, for all tables, the nucleotide values for each experimental run lie in corresponding positions.

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TABLE 5.—NICOTINAMIDE-ADENINE-DINUCLEOTIDE CONTENT OF RAT BRAIN

	(μmole per g of frozen brain)			
	Age in days			
	1	5	10	21
Experimental no.				
1	0·12	0·17	0·16	0·22
2	0·16	0·23	0·20	0·14
3	0·16	0·20	0·13	0·21
4	0·17	0·15	0·16	0·20
Mean ± 95% C.I.	0·15 ± 0·04	0·19 ± 0·06	0·16 ± 0·05	0·19 ± 0·06

Each value was obtained from pooled brain acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10-day, 3; 21-day, 2. Within each age group, for all tables, the nucleotide values for each experimental run lie in corresponding positions.

The choice of reference standard (see POPE, 1955) is especially difficult here because of the changing proportions in the developing rat brain of proteins, dry weight, lipids, and cell densities. We have calculated the high-energy phosphate concentrations with references other than 'frozen tissue weight', using average dry weights at different stages of development as determined in our laboratory and as taken from the literature and using defatted dry weights as published (FOLCH-PI, 1955; CLOUET, 1956). The general picture is one of maximal concentrations of high energy phosphates at 5 days of age and then a decline per unit weight with increasing age. For example, the ATP in μmoles per g of dry weight is 15·9 in the 1-day old rats, 18·8 in the 5-day, 15·9 in the 10-day, and 10·9 in the 21-day. However, when the concentrations of the high energy phosphates are calculated with DNA as a reference standard (DAHL, 1959) they increase with age. Further, if it is assumed that each cell contains 6·1 $\mu\mu$g DNA, then the ATP per cell increases from 4·7 μm-moles in the 1-day old rat to 7·0 μm-moles in the 21-day old rat brain. The concentration per unit of fresh weight seems to be the most useful reference, since fresh weight can be related easily to other standards. We have found that the weight of frozen tissue is very close to the fresh weight of the same piece of tissue. Therefore, all the values in the tables are presented on the basis of frozen tissue weight.

The ADP levels increase with maturation and may mean either an *in vivo* change with maturation or an artifact of the freezing process. A higher ATP/ADP ratio may be maintained in the 1-day rat by the greater ratio of mitochondria to high-energy phosphate utilization (SAMSON, 1960). On the other hand, the increased ADP in the 21-day animal and the correspondingly lower ATP/ADP ratio may be a result of increased freezing rate, for it must be remembered that the 1-day rat has no fur and weighs about 6 g whereas the 21-day rat has a full coat of fur and weighs about 40 g. When the rats are plunged into the liquid nitrogen, the freezing is rapid but not immediate, and is probably slower in the older animals. If the ATP were breaking down to ADP during the freezing, the ADP levels would be expected to increase with age. The ATP/ADP ratio in the 1-day rat then, would be a more accurate representation of an *in vivo* steady state. The AMP also increases progressively with age but is always less than 10 per cent of the ATP concentration.

TABLE 6.—CREATINE CONTENT OF RAT BRAIN

	(μMoles per g of frozen brain)			
	Age in days			
	1	5	10	21
Free creatine	3·12	3·22	4·12	5·62
	2·78	3·17	3·45	5·58
	2·38	3·24	3·83	6·50
	2·85	3·13	3·45	7·85
	3·12	3·04	3·95	7·75
	—	—	4·85	6·86
	—	—	3·66	—
Mean ± 95% C.I.	2·85 ± 0·38	3·16 ± 0·10	3·90 ± 0·45	6·69 ± 1·04
Total creatine	6·24	5·05	7·96	8·60
	5·11	6·05	6·40	8·18
	5·82	6·20	6·45	8·00
	6·04	5·75	6·20	9·25
	6·08	5·89	7·87	9·65
	—	—	7·00	9·55
	—	—	5·75	—
Mean ± 95% C.I.	5·86 ± 0·55	5·79 ± 0·55	6·80 ± 0·78	8·87 ± 0·75
Phosphoryl-creatine	3·12	1·83	3·84	2·98
	2·33	2·88	2·95	2·60
	3·44	2·96	2·22	1·50
	3·19	2·62	2·75	1·40
	2·86	2·85	3·92	1·90
	—	—	2·15	2·69
	—	—	2·09	—
Mean	2·99	2·63	2·84	2·18
Free Creatine/ Phosphoryl-creatine	1·00	1·76	1·07	1·89
	1·19	1·10	1·17	2·14
	0·69	1·09	1·81	4·33
	0·89	1·19	1·25	5·61
	1·09	1·07	1·01	4·07
	—	—	2·25	2·55
	—	—	1·75	—
Mean	0·97	1·24	1·47	3·43

Each value was obtained from pooled acid-soluble fractions. The number of brains pooled for each age group was: 1-day, 4; 5-day, 3; 10-day, 3; 21-day, 2.

The extraction procedures commonly used for the nucleotides and phosphoryl-creatine were developed largely for ATP and may not be adequate for the others. Indeed, some experiments can be cited from the literature (HEALD, 1957*a*,*b*) suggesting that guanine nucleotide in acid medium may be bound to a stable macromolecule which splits, at least partially, into its component parts at higher pH values. This line of reasoning might explain the variation found in the GTP levels (Table 2).

Inosine mono-, di-, and triphosphates and cytidine di-, and triphosphates are conspicuously absent. The total absence of the inosine compounds suggests a very low *in vivo* concentration. CMP was appreciable in all ages, and its appearance

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rather than CDP or CTP implies a rapid utilization of these compounds in the biosynthesis of products such as lipids (ROSSITER, 1960) at a rate greater than the corresponding rephosphorylation from ATP.

The roles of the triphosphates of guanosine, uridine, and cytidine in the energy flow have not been as thoroughly studied as that of ATP. However, the transfer of phosphate between ATP and these compounds makes possible the rapid distribution of energy from ATP, and ATP in turn can be quickly renewed by the reactions of the respiratory chain.

The major part played by most of these compounds in the physiology of development can hardly be more than speculated upon, but the knowledge of their concentrations is a beginning toward a better understanding of the chemistry involved.

SUMMARY

Nucleotides of adenine, guanine, uridine, and cytidine were investigated during neonatal maturation of the rat brain from 1 to 21 days.

Adenine, guanine, and uridine represented approximately 65, 20, and 15 per cent, respectively, of the total extractable nucleotides. The total high energy phosphate concentration did not change greatly during maturation although there was a slightly higher level on the 5th day. The ATP concentrations were relatively constant, while ADP and AMP increased with age. GTP and UTP levels did not change significantly during maturation. CMP represented the only detectable cytidine phosphate. Phosphoryl-creatine decreased with age, while the total creatine increased. NAD was present in significant concentrations at all ages.

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BRAIN CHOLESTEROL: BIOSYNTHESIS WITH SELECTED PRECURSORS *IN VIVO*

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OUR previous studies (KABARA *et al.*, 1957, 1958) on the biosynthesis and metabolism of cholesterol revealed that certain precursors were incorporated into the brain cholesterol of adult mice. Prior to these findings other workers reported low or almost undectable rates of lipid metabolism in the brain of adult animals (ROSSITER, 1957; MCILWAIN *et al.*, 1959). More recent studies have suggested that the adult mouse brain can synthesize cholesterol *in vivo* when the route of precursor administration is either intracisternal (MCMILLAN *et al.*, 1957) or intracerebral (NICHOLAS and THOMAS, 1959). However, only a small amount of incorporation was detected when precursors were administered intraperitoneally. In our initial studies on the *in vivo* incorporation of precursors into brain sterol we employed the intraperitoneal route of injection. Since our data appeared to differ from the results obtained by others, a more detailed investigation of our initial findings (KABARA and OKITA, 1959) was carried out.

MATERIAL AND METHODS

Radioactive precursors. With the exception of DL-[2-^{14}C]mevalonate which was generously supplied by Merck, Sharp, and Dohme Company, all of the radioactive precursors were purchased from either Tracerlabs Inc., or from New England Nuclear Corporation. The metabolites were dissolved in physiological saline to which benzyl alcohol (0·9%) was added as a preservative and the resulting solutions were injected intraperitoneally.

Experimental design. In the first series of experiments normal female CF-1 mice (9–12 weeks old) and normal female DBA/2 mice (10–12 weeks old) were injected with both a ^{3}H- and a ^{14}C-labelled precursor of cholesterol. In every case the tritium tagged molecule (10 mc/m-mole) was [2-^{3}H]acetate (400 μc/kg). The ^{14}C precursor (40 μc/kg) was one of the following: [1-^{14}C]acetate (2·8 mc/m-mole); DL-[3-^{14}C]leucine (0·6 mc/m-mole); uniformly labelled D-[^{14}C]glucose (0·23 mc/m-mole); and DL-[2-^{14}C]mevalonate (5 mc/m-mole). Two series of experiments were carried out in which all animals were starved for 24 hr before isotope injections. In the first instance animals were injected simultaneously with ^{3}H and ^{14}C-labelled precursors and killed 15 min later by decapitation and exsanguination. The organs were removed, washed twice in isotonic saline, and quick-frozen in a mixture of dry-ice and acetone. Samples were kept in the frozen state until required for analysis. In the second series of experiments the time course for the *in vivo* labelling of cholesterol following administration of [2-^{14}C]mevalonate was determined. Four groups of 10 mice each were injected with both [2-^{3}H] acetate and [2-^{14}C]mevalonate and killed at 10, 20, 40, and 80 min intervals. The tissues were removed and prepared for analysis as described above.

Isolation and assay of radioactive cholesterol. The colorimetric determination of cholesterol content and radioassay of isotope incorporation were made on the same tissue sample. The procedure for the isolation and assay of ^{14}C- and ^{3}H-labelled cholesterol was a modification of earlier methods (KABARA *et al.*, 1957). Briefly, plasma, red blood cell, or tissue cholesterol was extracted with acetone–alcohol (1:1); digitonin in 50% alcohol was added to precipitate the free sterol. The resulting filtrates were not analysed for esterified cholesterol. The digitonides resulting from the

precipitation of free cholesterol were dissolved in dioxane. Part was taken for a quantitative colorimetric determination and the rest was simultaneously assayed for ^{14}C and ^{3}H in a liquid scintillation counter (OKITA *et al.*, 1957). The resulting data are reported in terms of activity, DPM (disintegrations per min) per g wet tissue.

Radiochemical purity. In order to ascertain radiochemical purity of cholesterol isolated by our digitonide procedure, three groups of five mice each were injected with [1-^{14}C]acetate (80 μc/kg) and killed after 15 min. Free cholesterol was isolated from brain and liver and counted in the usual manner while the remainder of the sample was repurified via the dibromide derivative. After [1-^{14}C] acetate administration the initial specific activity of brain cholesterol before dibromide repurification was 1285 $\pm$ 42 DPM/mg and for liver cholesterol 12,918 $\pm$ 276 DPM/mg. After bromination

TABLE 1.—INCORPORATION OF VARIOUS PRECURSORS INTO FREE CHOLESTEROL OF BRAIN

Precursor (40 μc/kg)	CF-1 Female mice		
	Expt. 1.	Expt. 2.	Expt. 3.
	(disintegrations per min per g wet tissue)		
[1-^{14}C]acetate	198*	770	1319
[2-^{3}H]acetate	124	310	453
DL-[2-^{14}C]leucine	217	453	596
[2-^{3}H]acetate	120	710	293
D-[^{14}C]glucose†	2581	2797	3367
[2-^{3}H]acetate	354	288	503

* Each value represents weighted mean of five animals.
† Uniformly labelled.

and dibromination the specific activities were 1092 $\pm$ 95 and 11,686 $\pm$ 100 DPM/mg, respectively. Similar experiments were carried out with uniformly labelled [^{14}C]glucose (40 μc/kg). The initial specific activity of brain cholesterol was 940 $\pm$ 53 DPM/mg and of liver cholesterol 3309 $\pm$ 120 DPM/mg. After purification the specific activities were 1106 $\pm$ 68 DPM/mg and 3103 $\pm$ 180 DPM/mg. The differences noted for both [1-^{14}C]acetate and [^{14}C]glucose were well within the deviation to be expected during the purification procedure.

RESULTS

Labelling with selected precursors

In the first series of experiments a comparison was made of the efficiency of precursor incorporation into cholesterol. Normal adult mice were killed 15 min after intraperitoneal injection of labelled precursors; this was at or before the time of maximal incorporation for lipid labelling for the various precursors studied. The individual values in each experiment for [1-^{14}C]acetate, [2-^{3}H]acetate, DL-[2-^{14}C] leucine and uniformly labelled D-[^{14}C]glucose are given in Tables 1 and 2 and represent the weighted means for five mice.

Both normal CF-1 mice and normal DBA/2 animals incorporated the same amount of [1-^{14}C]acetate into brain cholesterol. The amount of radioactivity after [2-^{3}H] acetate administration was one-third that of [1-^{14}C]acetate incorporation.

The use of DL-[2-^{14}C]leucine was prompted by the increasing interest of this amino acid in the biosynthesis of sterols. The conversion of leucine into the cholesterol nucleus may proceed via two alternate pathways (COONS *et al.*, 1959). Evidence from

our respiratory carbon dioxide studies (KABARA and OKITA, 1959), as well as work of other investigators, seems to indicate a breakdown of the amino acid labelled in the carbon two position into [1-^{14}C]acetate and its subsequent reutilization as a two-carbon fragment. With this proposed metabolic scheme as a basis for comparison, the amount of radioactivity that appeared in brain cholesterol from [2-^{14}C]leucine represents only about 20 per cent of the expected radioactivity derived as an [1-^{14}C] acetate fragment. An even greater difference was found in the DBA/2 animals.

TABLE 2.—INCORPORATION OF VARIOUS PRECURSORS INTO FREE CHOLESTEROL OF BRAIN

Precursor (40 μC/kg)	DBA/2 Female mice		
	Expt. 1.	Expt. 2.	Expt. 3.
	(disintegrations per min per g wet tissue)		
[1-^{14}C]acetate	644*	685	828
[2-^{3}H]acetate	371	341	426
DL-[2-^{14}C]leucine	69	84	223
[2-^{3}H]acetate	247	582	—
D-[^{14}C]glucose†	3750	5020	5218
[2-^{3}H]acetate	528	1136	1238

* Each value represents weighted mean of five animals.
† Uniformly labelled.

Glucose, because of its importance as an energy source for nerve tissue, was also investigated as a possible precursor for brain cholesterol. The incorporation of radioactive glucose into brain cholesterol was greater than that of mevalonate, acetate, or leucine. This is in contrast to the more efficient incorporation of mevalonate into cholesterol of other tissues. The incorporation of [^{14}C]glucose into brain cholesterol was four to ten times greater than that of acetate.

In vivo *time course of cholesterol labelling with* [2-^{14}C]*mevalonate*

For the proper interpretation of our incorporation data of [2-^{14}C]mevalonate as a precursor for brain cholesterol, it became necessary to know the time of maximal incorporation after precursor injection. While mevalonic acid has been carefully studied in a variety of different experimental situations (LYNEN, 1959) the authors are not aware of any data on the time course of *in vivo* labelling of cholesterol by mevalonate in mice. Therefore, the time-course of incorporation of [2-^{14}C]mevalonate into free cholesterol of various tissues was determined.

When compared with acetate, liver sterol labelling from mevalonate was slower but greater (Fig. 1). The activity from [2-^{14}C]mevalonate reached its highest value between 40 and 80 min while maximal labelling of tissue sterol from [2-^{3}H]acetate (Fig. 2) occurred between 10 and 20 min. In tissues other than brain the incorporation of radioactive mevalonate into cholesterol was 25–39 times as great as that of acetate but in brain cholesterol, activity was higher with acetate as precursor than with mevalonate as precursor.

The difference in lipid labelling between acetate and mevalonate seems to indicate

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that an unusual metabolic situation exists in the brain. In this tissue the greatest degree of labelling with either precursor occurred in the first group killed 10 min after injection. The 'apparent turnover' of free cholesterol in the brain appears to be so rapid that only trace activity could be detected at the 80 min interval. Although the

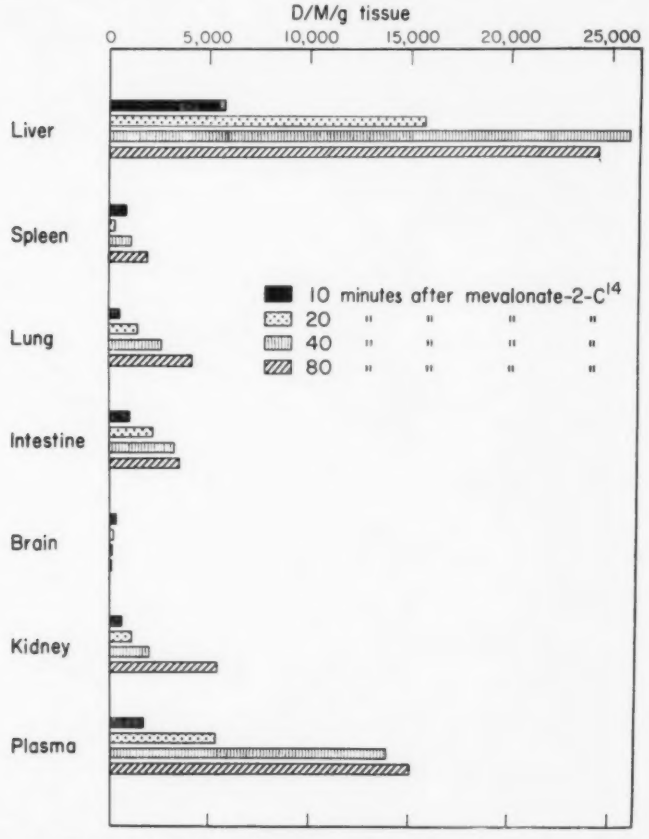

FIG. 1.—Tissue values for radioactivity free cholesterol. Radioactive mevalonate was injected intraperitoneally and the sterol isolated as a digitonide at various times after isotope injection.

biological half-life of this active brain free-cholesterol component was extremely fast, it should be emphasized that this component probably represents a small compartment and *does not necessarily reflect the turnover of total brain cholesterol.*

Whatever the ultimate mechanism of cholesterol biosynthesis in the central nervous system, the labelled sterol found in the brain was the result of local tissue synthesis and was not due to the transport of radioactive cholesterol from other regions. This is based on the evidence that the values for free cholesterol in plasma were *rising* while the specific activity of brain cholesterol was *decreasing*.

DISCUSSION

Since the cholesterol content of the human brain increases between infancy and adulthood from 2 g to 25 g (FOLCH-PI, 1955) there must be some mechanism to account

for this increase. Brain sterol may be a result of utilization of plasma cholesterol and/or local synthesis. Early experiments on brain lipids, however, indicated that the cholesterol of the adult brain is relatively metabolically inert. These studies indicated that there is apparently no exchange between brain cholesterol and plasma lipoprotein (BLOCH, 1948). Also WAELSCH *et al.* (1940) found no activity in the unsaponifiable fraction of brain lipids in adult rats given deuterium oxide. In agreement with these

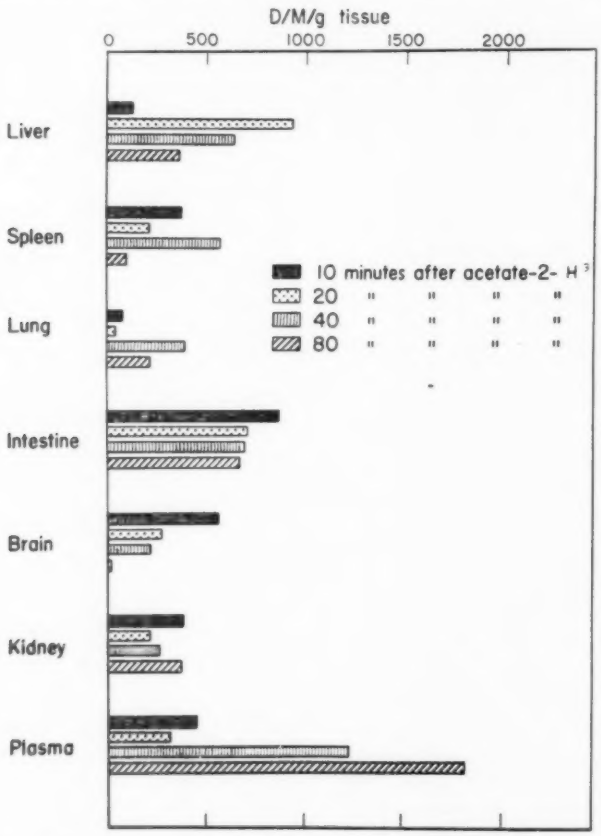

FIG. 2.—Tissue values for radioactive free cholesterol. Radioactive acetate was injected intraperitoneally and the sterol isolated as a digitonide at various times after isotope injection.

in vivo findings, others using *in vitro* techniques showed that [^{14}C]acetate can be readily converted into brain sterol in very young animals while surviving brain tissue slices of adult rats and rabbits cannot accomplish this conversion (SRERE *et al.*, 1949). The lipids of adult human brain tissue have been described as being relatively inert in terms of *in vitro* [1-^{14}C]acetate incorporation (AZARNOFF *et al.*, 1958).

In contrast to these earlier findings an active metabolism for brain cholesterol has been indicated where special experimental techniques are utilized. GROSSI *et al.*, (1958), using several different radioactive metabolites, demonstrated activity in brain slices for both young (10 day) and older (60 day) rats. *In vivo* data have also been provided by MOSER and KARNOVSKY (1959) on brain lipid synthesis in intact mature

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animals (6 months). Additional evidence for the biogenesis of brain cholesterol was obtained after intracisternal (McMillan *et al.*, 1957) and intracerebral injections (Nicholas and Thomas, 1959). Despite these findings there is still the general feeling that intraperitoneal injection of a precursor leads to little if any brain lipid labelling (Nicholas and Thomas, 1959) and that the negligible incorporation found after this type of injection reflects a lack of metabolite penetration into the brain rather than an inherent decrease in the synthetic capacity of this organ.

The data obtained from our studies indicate an active rather than static metabolism for free cholesterol in adult brain. A possible explanation for this demonstration of active *in vivo* brain cholesterol biogenesis in adult animals may be due (1) to our experimental techniques; (2) to the fact that our animals were killed shortly after labelled precursor injection; or (3) to our method of calculating the data. The last is most important since the high concentration of cholesterol in brain tissue makes the expression of experimental results in terms of specific activity (disintegration per min per mg) misleading. The initial radioactive cholesterol which forms in the brain shortly after isotope administration is diluted by a greater number of non-labelled sterol particles in this tissue than in other non-neural tissues. This dilution may be five or eight times greater for brain cholesterol than for sterol found in other organs. Moser and Karnovsky (1959) also noted that whereas the specific activity of brain lipids was low, the total activity incorporated into mature animals was significant.

Under the conditions of our experiments glucose proved to be a more efficient precursor than acetate, leucine, or even mevalonic acid. While mevalonate is a more immediate precursor of cholesterol than any of the other metabolites studied it was less efficient in the *in vivo* labelling of brain sterol than acetate. This apparent discrepancy may be due to difficulty in penetrating the blood-brain barrier or to the failure of cell penetration. Garattini *et al.* (1959) found that the efficiencies of labelling by [1-^{14}C]acetate and [2-^{14}C]mevalonate were more alike when brain homogenates were used. From these and our own data, it is evident that the efficiency of labelling cholesterol depends in part on the precursor used, the tissue under study, the experimental approach employed, *i.e.*, *in vivo* or *in vitro* methods, and in the case of the former method, the length of time beween administration of the precursor and destruction of the animal.

The persistence of labelled cholesterol in the mammalian central nervous system for over a year has been demonstrated by Davison *et al.* (1959), Davison and Wajda (1959), McMillan *et al.* (1959), and Nicholas and Thomas (1959). The rapid half-life obtained for free cholesterol in brain in our time-course experiments with [2-^{3}H] acetate and [2-^{14}C]mevalonic acid may reflect the turnover of an active, and probably a small, free cholesterol compartment in the brain. While the biological half-life for total brain cholesterol may be much longer, it could not be measured with the amount of isotope employed in our experiments owing to the rapid loss of labelled cholesterol from the active compartment. Available data seem to suggest that there is a series of sterol compartments in tissues, each with its individual turnover rate, and that the present experiments only emphasize the controversial nature of studies on adult brain cholesterol metabolism. Obviously more data will be necessary before a clearer biochemical picture emerges.

SUMMARY

Active brain cholesterol biogenesis in mice (9–16 weeks old) was demonstrated by

the incorporation of [1-^{14}C]acetate, [2-^{3}H]acetate, DL-[2-^{14}C]leucine, uniformly labelled D-[^{14}C]glucose, and DL-[2-^{14}C]mevalonate into free cholesterol in the brain within 15 min after intraperitoneal injection of the labelled precursor.

Of the precursors studied glucose was found to be incorporated into free cholesterol in the brain to the greatest extent.

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CHANGES IN AMINO NITROGEN DURING EEG DEPRESSION

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STUDIES of the metabolism of the brain during EEG depression have shown that the depression of cortical EEG activity is accompanied by a decrease in the glycogen and glucose content of the hemispheres (KŘIVÁNEK, 1958). There is also a decrease in the easily hydrolysable phosphorus and bound creatine and an increase in the lactic acid content (ZACHAR and RUŠČÁK, 1958). These observations agree with the findings of TSCHIRGI *et al.* (1957) who described local changes of pH during spreading cortical depression; these changes are similar to those described by DUBUISSON (1950) during muscle contraction and which are due to the breakdown of high-energy phosphates or to glycolysis. The relation of the metabolic changes to the EEG depression is not yet understood. It is probable that the decrease in glycogen and phosphocreatine and the increased lactic acid production during spreading depression result from increased neuronal activity, as has been suggested by GRAFSTEIN (1956); but the cause of the EEG depression is still unknown. New light has been thrown on this problem by experiments of VAN HARREVELD (1959), who has isolated from the brain a ninhydrin-positive substance, which produces bioelectrical changes similar to EEG depression, when applied locally to the cerebral cortex. In an investigation of the possible participation of the free amino acids of the brain in spreading depression, we have now determined the ninhydrin-positive substances in the cerebral hemispheres during EEG depression.

METHODS

The experiments were carried out with rats of about 200 g. A trephine hole was made in the skull under allobarbitone (Dial) narcosis and a piece of filter paper, dipped in a 25% KCl solution, was applied to the exposed cortex, to produce the EEG depression. After 30 min of the KCl application, when on the average 7 waves of depression had passed in the hemispheres (RUŠČÁK and DUDA, 1959), the brains were fixed in liquid air. After the craniotomy over the whole dorsal surface of both hemispheres, dura mater was removed and the cortex was dissected in liquid air. After grinding to a powder in a mortar, the tissue was extracted three times with 10 vol. of 80% ethanol acidified with acetic acid to pH 3. In the extracts obtained the free amino nitrogen was determined according to TROLL and CANNAN (1953) and some free amino acids were determined by one-dimensional paper chromatography, using as the solvent *n*-butanol–formic acid–water (75:15:10 by vol.). The quantitative evaluation of amino acids was carried out according to FISCHER and DÖRFEL (1953).

After extraction of the tissue with 80% ethanol, the nitrogen in the residue was determined by the micro-Kjeldahl distillation method. The results are expressed as μmoles amino nitrogen or amino acid per g of nitrogen in the residue insoluble in 80% ethanol.

RESULTS

In control experiments on 17 animals without EEG depression no significant difference was found in the free amino nitrogen in the right and the left hemispheres

expressed as μmoles per g nitrogen in the ethanol extracted tissue residue. During spreading depression, caused by the action of KCl, a significant increase in free amino nitrogen occurred in the experimental hemispheres; the free amino nitrogen increased from 2024 μmoles to 2311 μmoles per g insoluble nitrogen ($P < 0{\cdot}01$). A decrease in aspartic acid, glutamic acid and glutamine, and an increase in alanine, were observed. These changes were not statistically significant (Table 1.)

TABLE 1.—CHANGES IN AMINO NITROGEN AND SOME AMINO ACIDS DURING SPREADING EEG DEPRESSION

	Carotids free		Carotids tied	
	Mean ± S.D.	statistical data	Mean ± S.D.	statistical data
Amino nitrogen	C 2024 ± 218	$n = 30$	C 3013 ± 301	$n = 23$
	D 2311 ± 247	$P < 0{\cdot}01$	D 2675 ± 34	$P < 0{\cdot}01$
Aspartic acid	C 263 ± 56	$n = 9$	C 291 ± 74·7	$n = 33$
	D 256 ± 50	$P > 0{\cdot}5$	D 238 ± 41·2	$P < 0{\cdot}01$
Glutamic acid	C 444 ± 49	$n = 9$	C 361 ± 57·2	$n = 34$
	D 420 ± 42	$P < 0{\cdot}5$	D 289 ± 55·2	$P < 0{\cdot}01$
Glutamine	C 360 ± 39	$n = 9$	C 304 ± 38·5	$n = 33$
	D 330 ± 44	$P < 0{\cdot}5$	D 253 ± 44·7	$P < 0{\cdot}01$
Alanine	C 46·1 ± 8·5	$n = 9$	C 48.5 ± 6·8	$n = 34$
	D 47·9 ± 6·3	$P > 0{\cdot}5$	D 111 ± 15·6	$P < 0{\cdot}01$

Results as μmoles/g nitrogen in ethanol extracted tissue. C = control hemisphere. D = hemisphere with EEG depression.

In a further series of experiments the spreading depression was evoked in the brain of rats, in which both carotids were tied. It was expected that, similar to the changes in phosphocreatine and lactic acid, reported earlier (RUŠČÁK and DUDA, 1959) the changes in free amino nitrogen would become more evident. In these experiments a significant decrease in free amino nitrogen occurred in the experimental hemisphere in comparison with the control hemisphere, although the ninhydrin-positive nitrogen in both hemispheres increased in comparison with that in the experiments with untied carotids. Simultaneously, the depressed hemispheres showed a statistically significant decrease in aspartic acid, glutamic acid and glutamine and an increase in a ninhydrin-positive substance, whose mobility in the solvent system used was identical with alanine. A summary of the results, evaluated statistically by Student's t-test, is given in Table 1.

DISCUSSION

During EEG depression, the ninhydrin-positive fraction of the brain was metabolically active. The increase in this fraction may be due to an increase in ammonia alone, or in ammonia together with peptide components. The observed decreases in aspartic acid, glutamic acid and glutamine, especially after tying the carotids, may be

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due to their increased utilization by nervous tissue during oligæmia, when the supply of metabolites to the brain via the bloodstream is limited. But it is also possible, that these changes in the contents of free amino acids in the brain are directly connected with the EEG depression. This is supported by the experiments of BERL *et al.* (1959) who found that cooling the surface of the brain, which is accompanied by EEG changes of an epileptiform type, caused decreases in the contents of glutamic acid, glutamine and glutathione. The decrease in the ninhydrin-positive components observed by BERL *et al.* (1959) was only found under the influence of cold; prolonged electrical stimulation of the sensorimotor area of the brain for 2 hr did not affect the metabolites mentioned. Since cold can produce EEG depression (ZACHAROVÁ and ZACHAR, 1961) it is probable that the changes of amino acids described in our experiments are due to EEG depression.

The increase in alanine in EEG depression, after tying the carotids, may be due to reductive amination of pyruvic acid, arising from the increased glycolysis (RUŠČÁK and DUDA, 1959), either by transamination from glutamic acid or by direct amination without participation of glutamic acid. This possibility is suggested by the observations of BEREZOVSKAYA (1958), who described the amination of pyruvic acid from ammonium salts by liver mitochondria. It may be that the increase in the alanine fraction is due to β-alanine, which might be formed by the decarboxylation of aspartic acid. As an ω-amino acid—together with GABA, which was also increased in these experiments (unpublished results),— it might cause the disappearance of the spontaneous and the evoked electrical activity (PURPURA *et al.*, 1959). This is also found for spreading EEG depression.

SUMMARY

A statistically significant increase in ninhydrin-positive substances in rat brain was found during spreading EEG depression, produced by potassium ions. During oligæmia of the brain, caused by tying both carotids, the potassium evoked EEG depression brought about a decrease in ninhydrin-positive substances, in comparison with the contralateral control hemisphere. A statistically significant decrease in glutamine, glutamic acid and aspartic acid and an increase in the fraction, whose mobility in the system *n*-butanol–formic acid–water (75:15:10 by vol.) was identical to that of alanine, were also observed. The possible relation of these changes in ninhydrin-positive substances to spreading EEG depression is discussed.

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THE SYNTHESIS OF CREATINE BY THE BRAIN OF THE INTACT RAT*

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CREATINE synthesis was first demonstrated by BORSOOK and DUBNOFF (1940) in experiments in which rat liver slices were incubated with guanidoacetic acid and methionine. In subsequent studies, DUVIGNEAUD (1941, 1956) demonstrated that the methyl group of methionine is transferred as a unit to guanidoacetic acid to form creatine *in vivo*. BLOCK and SHOENHEIMER (1941), using [^{15}N]-labelled compounds, and BORSOOK and DUBNOFF (1941) using kidney slices and cell free extracts, later demonstrated guanidoacetic acid synthesis from arginine and glycine. BORSOOK and DUBNOFF (1941) proposed a mechanism, accommodating all of these findings, whereby the total body requirement of creatine might be synthesized in animals. The synthesis of creatine was tentatively described as proceeding in two generalized steps:

$$\text{Arginine} + \text{glycine} \xleftrightarrow[\text{transamidinase}]{\text{glycine}} \text{guanidoacetic acid} + \text{ornithine} \qquad (1)$$

$$\text{Guanidoacetic acid} + \text{methionine} \longrightarrow \text{creatine} + \text{homocysteine} \qquad (2)$$

It was concluded by these authors that the first step occurred only in kidney, catalysed by the enzyme glycine transamidinase, which was thought to be peculiar to that organ and which could not be demonstrated in a variety of tissues including brain. The second reaction was described as occurring in liver. Its enzymic aspects have since been more completely worked out (CANTONI and VIGNOS, 1954) and it is now thought that an activated form of methionine (*S*-adenosylmethionine), the product of the reaction of adenosine triphosphate (ATP) and methionine (CANTONI and DURELL) 1957), contributes its methyl group to guanidoacetic acid by the action of the enzyme guanidoacetic acid methylpherase to form creatine and adenosylhomocysteine.

Recent studies (SANDBERG, HECHT and TYLER, 1953) suggested that the earlier formulation of organ specificity in creatine synthesis may be incorrect. Evidence for major extrarenal transamidination was presented by WALKER (1958) who demonstrated arginine-glycine transamidinase activity in dog pancreas and who also showed the activity of this enzyme to be five times that of renal transamidinase. HORNER (1959) showed that nephrectomized rats were capable of significant extrarenal transamidination and creatine synthesis. With special regard to brain, PISANO and UDENFRIEND (1958) reported the synthesis of both guanidoacetic acid and γ-guanidobutyric acid in brain homogenates incubated with arginine and [2-^{14}C]glycine. As yet, however, there has been no definitive demonstration of creatine biosynthesis in brain.

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† Scottish Rite Summer Medical Student Research Fellow.

The unique position phosphocreatine occupies in relation to energy metabolism as a storage source of high energy phosphate for the regeneration of ATP has been described for a number of tissues (LIPMANN, 1941). A more recent paper (ENNOR and MORRISON, 1958) dealing with the biochemistry of *N*-phosphorylated compounds in several biological systems, re-emphasized this. The importance of creatine in brain was pointed out by MCILWAIN (1957) in his discussion of the creatine-creatine phosphokinase-ATP system, and recently the question has arisen as to whether the brain has the capacity for creatine synthesis or whether it is dependent upon extracerebral sources for this compound.

As an extension of studies in this laboratory concerned with amino acid metabolism in brain (SPORN, DINGMAN and DEFALCO, 1959*a*; SPORN *et al.*, 1959*b*), the capacity of the brain to synthesize creatine from arginine was investigated by similar methods.

METHODS

Determination of creatinine. All determinations of creatinine were made with the alkaline picrate reaction (Jaffé) as modified by OWEN (1954). It was not necessary to use Lloyd's reagent as was suggested for the purpose of eliminating picrate reacting chromogens in serum, plasma, and urine. All colorimetry was done on a Bausch and Lomb 'Spectonic 20' Model spectrophotometer at a wave length of 520 mμ. Creatinine spots on chromatography paper were developed by spraying with a solution of picric acid in alcoholic NaOH. (1·3% picric acid in 80 ml 95% EtOH and 20 ml 10% NaOH). (BLOCK, DURRUM and ZWEIG, 1955).

Radiopurity of L-[^{14}C]*arginine.* The L-[^{14}C]arginine-monohydrochloride was recrystallized twice from aqueous acetone and assayed for radiopurity by chromatography on butanol:acetic acid:water (60:15:25) followed by radio-scanning. The isotope contained 2·44 per cent radio-impurities in two positions separate from a single ninhydrin spot corresponding to arginine. To eliminate the possibility that the impurity might be creatine or creatinine, a portion of the isotope was added to 10 mg of inert carrier creatine in 10 ml of N-HCl, and creatinine was isolated as described below by column and paper chromotography. Subsequent assay showed no radioactivity in the creatinine. Insomuch as the R_F of the radio impurities did not correspond to the R_F's of several organic guanidine compounds, including guanidoacetic acid, the possibility that the injected isotope contained creatine intermediates is unlikely.

Determination of radioactivity. All samples were counted at infinite thinness at 1250 V with a gas flow counter fitted with an end window. Determinations were done in duplicate, and each sample was counted for no less than 2000 counts. Correction for background radiation was made before calculation of specific activity.

Isolation and purification of creatinine. Four male albino rats (Sprague-Dawley strain) were injected as follows: rats 1 and 3 were injected intracisternally with 2 mg of uniformly labelled L-[^{14}C]arginine-monohydrochloride (Nuclear-Chicago Corp.) in a volume of 0·01 ml; 5 min after the intracisternal injection these same rats received an injection, in the exposed femoral vein, of 2 mg of inert L-arginine-monohydrochloride. Rats 2 and 4 (littermates, respectively, of rats 1 and 3) were injected intracisternally with 2 mg of inert L-arginine-monohydrochloride, and 5 min later were injected, in the exposed femoral vein, with 2 mg of uniformly labelled L-[^{14}C]arginine-monohydrochloride. All injections were performed under light ether anæsthesia with a microsyringe calibrated to 0·0001 ml, and the animals were alert and walking within 5 min following the intracisternal injection. The animals were decapitated exactly 30 min after receiving the injection of [^{14}C]arginine. The brains were quickly removed, cleaned of adhering blood and meninges, and homogenized in a Potter-Elvehjem homogenizer in 8 ml of 5% trichloroacetic acid (TCA). The blood obtained at decapitation was added to oxalated containers and centrifuged to obtain plasma. Plasma (2 ml) was added to 5 ml of 5% TCA. Inert carrier creatine (10 mg) was added to the brain homogenates and TCA treated plasma and the TCA concentration was brought up to 10 per cent. The samples were centrifuged; the supernatant solution was decanted and the precipitate was washed twice with 5 ml of 10% TCA. The washings and the original supernatant fluids were pooled as the acid soluble fraction and treated three times with 25 ml of CCl_4. Celite Analytical Filter Aid (8 ml) was added to the acid soluble fraction; the mixture was centrifuged,

and the supernatant solution poured off. The Celite was washed twice with water, and the supernatant solutions were pooled, taken down almost to dryness on a rotary evaporator, and then made up to 10 ml with N-HCl. This was boiled slowly under reflux for 4 hr to cyclize all creatine to creatinine. The solution was again taken to dryness and made up to 1 ml in N-HCl. Creatinine was isolated and purified in four sequential steps. Initially the samples were placed on 50 cm × 4 cm^2 columns of Dowex 50-X_4 resin equilibrated with N-HCl, and eluted in 5 ml fractions with N-HCl at a flow rate of 30 ml per hr. The creatinine peak was determined, and the corresponding solution was concentrated to a volume of less than 1 ml. A portion was removed and chromatographed on Whatman no 3. paper with butanol : acetic acid : water (60 : 15 : 25). The creatinine thus isolated from brain and plasma samples was eluted from that area which corresponded to the R_F of known samples of inert creatinine which were chromatographed adjacent to the samples. Specific activity of creatinine was determined by assaying the eluate for creatinine and radioactivity. Purification of creatinine to constant specific activity was accomplished by chromatography of the eluate from the first paper system in a pyridine : acetic acid : water (50 : 35 : 15) system followed by chromatography of the eluate from this system in methanol : pyridine : water (80 : 4 : 20).

TABLE 1.—RADIOACTIVITY IN ACID SOLUBLE FRACTIONS OF BRAIN AND BLOOD OF RATS KILLED 30 MIN AFTER INJECTION OF L-[^{14}C]ARGININE MONOHYDROCHLORIDE

Brain wet weight (g) / Total activity	Rat 1	Rat 2	Rat 3	Rat 4
	1·625	1·675	1·765	1·705
Injected arginine (counts/min/rat)	370 × 10^5	370 × 10^5	340 × 10^5	340 × 10^5
Plasma acid-soluble fraction (counts/min/2 ml)	1·6 × 10^5	0·93 × 10^5	8·6 × 10^5	0·81 × 10^5
Brain acid-soluble fraction (counts/min/brain)	84 × 10^5	0·53 × 10^5	86 × 10^5	0·72 × 10^5

Rats 1 and 3: intracisternal injection of labelled arginine.
Rats 2 and 4: intravenous injection of labelled arginine.

RESULTS

The levels of labelled creatinine reported for plasma and brain (Table 1) and used in the determination of specific activity, represent total brain and plasma creatine plus creatinine. After cyclization of the acid-soluble fractions, creatinine was found in concentrations of 3·3 mg per 100 ml of plasma, and 1 mg per g wet weight of brain.

Specific activity of brain creatinine was determined by multiplying the specific activity of isolated creatinine by $\frac{8{\cdot}6 + \text{creatinine in brain after cyclization}}{\text{creatinine in brain after cyclization}}$ where 8·6 is mg of recoverable creatinine after cyclization of 10 mg carrier creatine. Plasma creatinine values were determined by multiplying the specific activity of isolated creatinine from 2 ml of plasma by $\frac{8{\cdot}6 + 0{\cdot}066}{0{\cdot}066}$, where 8·6 is mg of recoverable creatinine, and 0·066 is mg of creatinine recoverable after cyclization of 2 ml plasma.

The specific activity of brain creatinine (Table 2) in those animals receiving L-[^{14}C]arginine intracisternally (i.c. animals) was at least twenty times as great as that isolated from the brains of the animals receiving the labelled arginine intravenously (i.v. animals). On the other hand, the specific activity of plasma creatinine

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was about 3–5 times as great in the i.v. animals as in the i.c. animals. In comparing the distribution of labelled creatinine between brain and plasma in the same animals, it was found that the specific activity of creatinine in the brains of the i.v. animals was only one twentieth of that of the plasma, while in the i.c. animals the specific activity of brain creatinine was about 4 times that of the plasma activity.

TABLE 2.—SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF CREATININE* ISOLATED FROM BLOOD AND BRAIN

	Rat 1	Rat 2	Rat 3	Rat 4
Specific activity of plasma creatinine (counts/min/mg)	580	1500	310	1500
Specific activity of brain creatinine (counts/min/mg)	1900	80	1400	70
Total radioactivity in plasma creatinine (counts/min/2 ml plasma)	410	1000	220	1000
Total radioactivity in brain creatinine (counts/min)	3100	140	2200	110

* Values of creatinine represent creatine plus creatinine present in brain or blood before cyclization.
Rats 1 and 3: intracisternal injection of labelled arginine.
Rats 2 and 4: intravenous injection of labelled arginine.

DISCUSSION

With this design two criteria must be satisfied before it can be concluded that brain is capable of creatine synthesis. First, it must be shown that the specific activity of creatine in the brains of the animals receiving an intracisternal injection of L-[^{14}C]-arginine (i.c. animals) is greater than that of the animals receiving an intravenous injection of L-[^{14}C]arginine (i.v. animals). Secondly, it must be shown that the level of labelled creatine in the brains of the i.c. animals does not arise as a result of (a) transfer of extra-cerebrally synthesized creatine across the blood brain barrier into the brain, (b) a slower clearance of extracerebrally synthesized creatine from brain than from blood, resulting in a relatively high level of labelled creatine in brain while that of blood is either very low or absent altogether, (c) direct contamination of brain by labelled creatine and creatinine present in cerebral blood at the time of decapitation.

Since the specific activity of brain creatinine in the i.c. animals is twenty times that in the i.v. animals the first criterion is satisfied. With regard to the second set of conditions, if it is assumed that all of the creatine is synthesized extracerebrally and that the creatine was transferred from blood to brain, then one should find the specific activity of brain creatinine to be lower than, or at best, equal to that of blood creatinine in both i.c. and i.v. animals. The data do not support this argument. If, as stated in (b) above, the clearance of creatine in brain was slower than that in blood in both i.c. and i.v. animals, then one would expect to find the specific activity of brain creatine to be higher than blood creatine in all animals regardless of

the route of injection of the L-[^{14}C]arginine. Again the data do not support this possibility. Finally direct contamination can be eliminated by considering that only 0·9 per cent of the total blood volume is present in brain of an exsanguinated rat (SHARPE, CULBRETH and KLEIN, 1950). This, along with observations of actual blood levels of labelled creatinine allows for a maximum of 10 counts per min in the whole brain that could arise from creatine plus creatinine in cerebral blood.

The data presented in Table 2 indicate that brain is capable of creatine synthesis. In the light of this finding, it would be of interest to determine the physiological significance of the pathway in brain.

SUMMARY

Evidence that the living rat brain can synthesize creatine from L-arginine is presented. In addition, a method of studying *in vivo* brain metabolism is discussed.

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γ-AMINOBUTYRIC ACID AND ITS ACTION ON THE ADRENAL CORTEX—I

EFFECTS OF γ-AMINOBUTYRIC ACID ON THE ASCORBIC ACID CONTENT OF THE ADRENAL GLAND

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SINCE ROBERTS *et al.* (1950) and AWAPARA *et al.* (1950) found that a large amount of γ-aminobutyric acid (GABA)* is contained in the mammalian brain, many biochemical and physiological studies on GABA have appeared. These investigations have brought to light properties of GABA such as its inhibitory action in the central nervous system (FLOREY, 1954*a*, *b*), its central blood pressure reducing function (TAKAHASHI *et al.*, 1958), and effects on the energy metabolism of the brain (MORI, 1958). In addition, our present studies made it clear that GABA markedly affects the adrenal cortex.

MATERIALS AND METHODS

Experimental animals. White rabbits of both sexes weighing about 3 kg each were used.

GABA. GABA, a product of Daiichi Seiyaku Co., Ltd., was recrystallized three times so that its m.p. was 202°. GABA was dissolved in distilled water until its concentration reached 250 mg/ml. This solution was injected into the auricular vein of the rabbit.

Adrenalectomy and extraction of ascorbic acid from the adrenal. The rabbit was laid on its back and fastened to the operating table, mid laparotomy was performed with ether anaesthesia, and the left adrenal was removed. Next, the solution of GABA or of the other substances used were injected into the auricular vein. At intervals after the injection (see below), the right adrenal was removed. Care was taken to prevent undue mechanical stimulation or injury of the adrenal. The blood of the excised adrenal was wiped off gently with a piece of gauze. After weighing the specimen, it was ground in 40 vols. of 2% metaphosphoric acid, and the ascorbic acid was extracted from the suspension. The amount of ascorbic acid was calculated by FUJITA's method (1953) involving the use of 2:6-dichlorophenolindophenol.

RESULTS

(1) Comparison was made of the ascorbic acid contents of the right and the left adrenals in control uninjected animals. Ten minutes after the removal of the left adrenal, the right adrenal was removed. There was clearly no difference between their ascorbic acid concentrations. (Fig. 1).

(2) Effect of GABA: GABA was injected intravenously (1·5 m-moles per kg body weight) into rabbits from which the left adrenal had been removed. At intervals of 10, 30, and 60 min after injection, the right adrenal was removed and the ascorbic acid content was determined. The results show (Fig. 1, 2–4) a remarkable reduction of adrenal ascorbic acid content at 10 min after the injection. At 30 min, a slight reduction remained, and at 1 hr the ascorbic acid concentration had returned to normal.

* Abbreviations used are: GABA, γ-aminobutyric acid; GABOB, γ-amino-β-hydroxybutyric acid.

We also measured the ascorbic acid content of the adrenal 10 min after an injection of a smaller amount of GABA (0·7 m-moles per kg body weight) and found a smaller reduction (Fig. 1, 5).

(3) Effect of hypophysectomy: after hypophysectomy the ascorbic acid concentration in the adrenals was 20 per cent less than normal, but no differences could be observed between the left and the right adrenals (Fig. 2, 1).

An injection of GABA (1·5 m-moles per kg body weight) was made into the veins of four hypophysectomized rabbits and the ascorbic acid content of the adrenals was measured. There was no change (Fig. 2, 2).

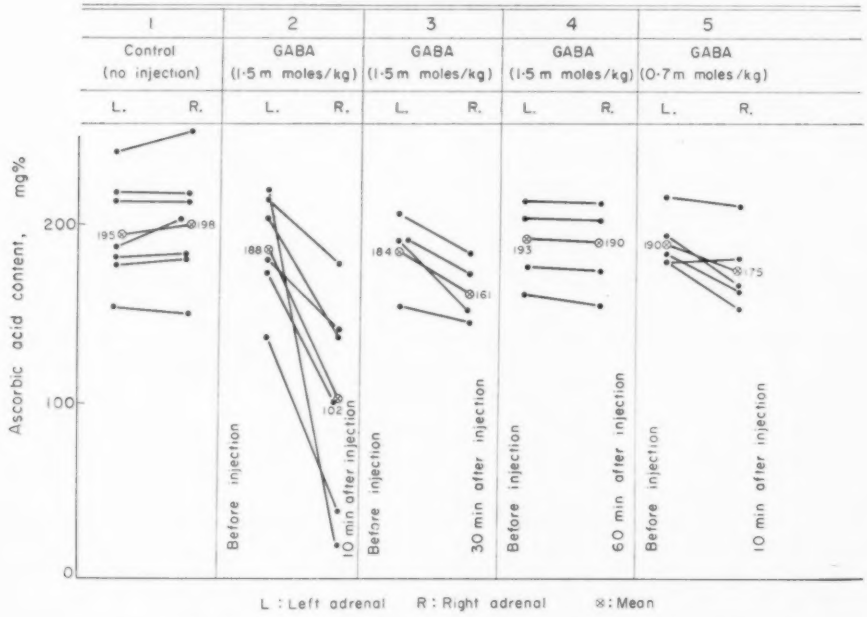

FIG. 1.—Ascorbic acid content of adrenals before and after injection of GABA

(4) Effect of amino acids or amines on the ascorbic acid content: with the same procedure as that used for GABA, several amino acids and amines were tested. Glycine, β-alanine, glutamic acid, γ-amino-β-hydroxybutyric (GABOB), aspartic acid, and asparagine were injected at a concentration of 1·5 m-moles per kg body weight. Histamine and *n*-butylamine were also injected at concentration of 0·5 μg and 10 mg, respectively per kg body weight. There was no reduction in adrenal ascorbic acid content such as had occurred after the GABA injections (Fig. 3, 1–8).

DISCUSSION

Recently, GABA has been used increasingly for the medical treatment of hypertension, for treatment of the mentally retarded, and for awakening from states of coma (AZUMA *et al.*, 1959; TAKAHASHI *et al.*, 1959; YAMAGUCHI, 1955; MURAKAMI, 1959). Basic studies of the effects of GABA on physiological mechanisms in relation to these clinical effects are now in progress. Our finding that GABA has a transitory effect in reducing the ascorbic acid content of the adrenals may be relevant in this connection.

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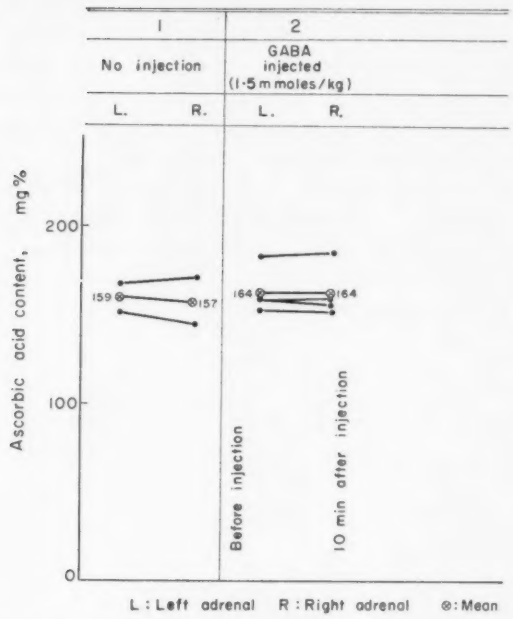

FIG. 2.—Ascorbic acid content of adrenals of hypophysectomized rabbits.

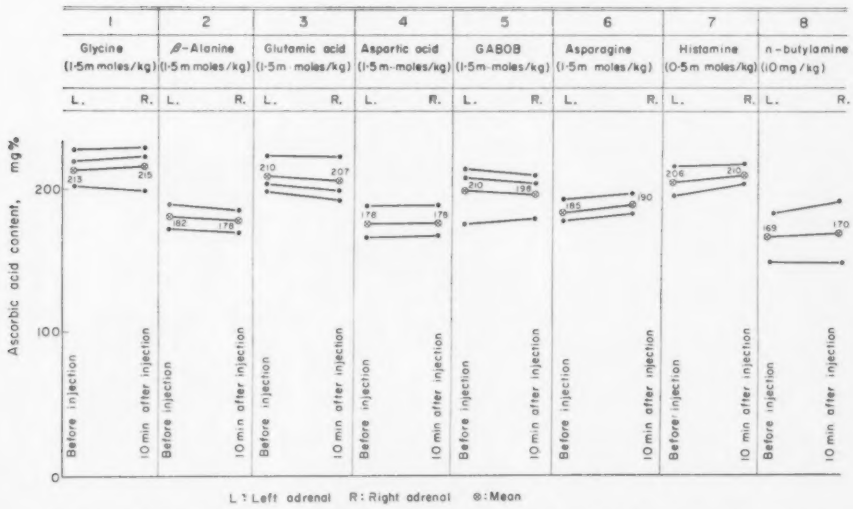

FIG. 3.—Ascorbic acid content of adrenals before and after injection of amino acids or amines.

In our experiments GABA was injected at a concentration of 1·5 m-moles (154·7 mg) per kg body weight; GABA is used clinically for adult patients at the rate of 3–20 g per day per person, equivalent to 50–300 mg per kg body weight. Therefore, the quantity of GABA used in our experiments cannot be regarded as especially large. The

injection of GABA at the lower concentration of 0·7 m-moles per kg body weight resulted in little reduction of adrenal ascorbic acid in our experiments.

We also studied the return of the ascorbic acid level of the adrenals to normal after injections of GABA. Normal values were nearly regained in 30 min and recovery was complete in 60 min. We can, therefore, say that the effect of GABA on the ascorbic acid content of adrenal is transitory.

GABA had no effect on adrenal ascorbic acid in rabbits which were hypophysectomized. Therefore, GABA appears to exert its effect on the adrenal via the hypophysis.

Finally, the injection of several amino acids and amines produced no reduction of adrenal ascorbic acid, in contrast to the effect of GABA.

At present we are investigating changes in adrenal corticoid metabolism following injections of GABA. This work will be described in a later paper.

SCHWARTZ and KLING (1960) reported a significant depletion of adrenal ascorbic acid under the stress of acute unilateral adrenalectomy in the cat, but this was not observed after drug injection. Similar results were reported by OHLER *et al.* (1956) in the cat and by SAYERS and SAYERS (1947) in the rat. In contrast in the present studies no adrenal ascorbic acid depletion was observed following laparotomy under ether anaesthesia in normal white rabbits. The discrepancy may be due to species differences.

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SUMMARY

(1) Intravenous injection of GABA in rabbits at a concentration of 1·5 m-moles per kg results in a large reduction of adrenal ascorbic acid concentration.

(2) This effect is transitory. The original value was nearly regained 30 min after the injection.

(3) This effect of GABA does not appear in hypophysectomized rabbits.

(4) Injections of such amino acids as glycine, β-alanine, glutamic acid, aspartic acid, γ-amino-β-hydroxybutyric acid and asparagine, and such amines as histamine and *n*-butylamine, caused no reduction of adrenal ascorbic acid.

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PROTEINASE ACTIVITY OF NERVOUS TISSUES IN ORGANO-PHOSPHORUS COMPOUND POISONING

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THE TOXIC effects of organo-phosphorus compounds in various animals have been known for many years. More recently it has been found that some of these poisons which are well known as anticholinesterases, such as tri*ortho*cresyl phosphate (TOCP), di*iso*propyl fluorophosphonate (DFP) and Mipafox or *NN'*-di*iso*propyl-phosphorodiamidic fluoride, were able to produce, notably in chickens, myelin degeneration with subsequent paralysis (SMITH, ELVOVE and FRAZIER, 1930; DAVID, 1954; EARL and THOMPSON, 1952; BARNES and DENZ, 1953; DAVISON, 1953) although the mechanism of action is still unknown.

Because of previous results on the changes of nerve tissue proteolytic activity during demyelination after transection (PORCELLATI and THOMPSON, 1957; PORCELLATI and CURTI, 1960) it seemed of interest to study the effect of DFP and TOCP on the neutral proteinase (ANSELL and RICHTER, 1954) of nervous tissues *in vivo*, during demyelination produced by these two anticholinesterases.

EXPERIMENTAL

Fully grown hens of mixed breed weighing between 1·8 kg and 2·5 kg, and held on a stock diet, were used throughout; 20 were used as control, 40 were treated with TOCP, and another 40 with DFP. The animals were fasted for 12–16 hr, poisoned by a single dose of TOCP or DFP, and killed on the 4th, 12th, 16th, 20th, or 30th day after poisoning. At the various intervals, 8 hens from each experimental group were killed by decapitation. TOCP was given undiluted by mouth (1 ml/kg body wt). DFP, suitably diluted, was injected subcutaneously (1 mg/kg body wt). Each bird was injected at least once with atropine sulphate (1 mg/kg body wt), immediately after treatment with TOCP or DFP, in order to suppress the muscarinic symptoms in the acute stages of poisoning. The brain, spinal cord, sciatic nerves and liver were quickly removed, washed free from blood in saline, blotted dry, cleaned of adherent fatty and connective tissue, diluted 1:4 in Krebs phosphate saline solution, Ca^{++} free, (buffered with 0·1 M-phosphate buffer pH 7·4), and homogenized in an all-glass Potter-Elvehjem homogenizer. The liver tissue was examined to determine whether the poisoning effects were similar to those of nerve tissues. The incubation mixtures, in a final volume of 3 ml, were held at 25° for 25 min, before being incubated under nitrogen in Warburg vessels, so that the results could be compared with those of *in vitro* experiments (MILLO and PORCELLATI, in preparation). The proteinase activity was determined, as described previously (PORCELLATI and CURTI, 1960). Duplicate analyses were made in each experiment and the results are expressed as μmoles NH_3–N per 100 g wet wt per hr.

In the sciatic nerve experiments no correction was made for the increase in water content (oedema), as WEBSTER (1954) reported that it did not occur during demyelination by TOCP. Histological investigations on nervous tissues from both poisoned and normal birds, were made by the GLEES modification of the Marchi staining method (PEARSE, 1960) and the pseudo and true cholinesterase levels in plasma, brain and spinal cord were determined according to the procedure of EARL and THOMPSON (1952).

RESULTS AND DISCUSSION

With either DFP or TOCP, paralysis was produced regularly in hens, 12–14

days after poisoning. Results of control experiments on the effects of these poisons on the cholinesterases, as well as the histological findings, have confirmed the results of previous authors (EARL and THOMPSON, 1952; BARNES and DENZ, 1953; DAVISON, 1953, and AUSTIN and DAVIES, 1954), thus showing the efficacy of these poisons in producing cholinesterase inhibition and demyelination in sciatic nerves and in some tracts of spinal cord.

The formation of NH_2-groups from the tissue proteins of hen's spinal cord, sciatic nerve, brain and liver, at 4, 12, 16, 20 and 30 days after intoxication are shown in Table 1.

TABLE 1.—THE PROTEINASE ACTIVITY OF HEN SPINAL CORD, SCIATIC NERVE, BRAIN AND LIVER DURING TOCP AND DFP POISONING

(Tissue preparations incubated for 60 min at 37° in Krebs phosphate-saline solution, pH 7·4, gas phase N_2. Mean of 8 estimations, except for normal tissues, where the mean is of 20 estimations.

Days after poisoning	Poison	Tissue examined			
		Spinal cord	Sciatic nerve	Brain	Liver
		μ-moles NH_2-N per 100 g wet wt per hr (mean ± S.E.M.)			
0	—	827 ± 48	579 ± 55	1124 ± 75	2998 ± 200
8	TOCP	925 ± 50	541 ± 43	1055 ± 119	1766 ± 313
	DFP	882 ± 90	626 ± 55	1096 ± 69	2067 ± 238
12	TOCP	1068 ± 112	786 ± 79	1209 ± 94	2196 ± 466
	DFP	704 ± 62	662 ± 70	1145 ± 41	2024 ± 226
16	TOCP	1106 ± 88	929 ± 55	1199 ± 82	2250 ± 401
	DFP	1048 ± 87	711 ± 70	1028 ± 104	1757 ± 207
20	TOCP	1174 ± 81	719 ± 40	1081 ± 106	2336 ± 433
	DFP	925 ± 91	823 ± 86	1037 ± 105	1503 ± 164
30	TOCP	778 ± 76	566 ± 42	1117 ± 104	2238 ± 166
	DFP	818 ± 81	574 ± 58	1078 ± 95	1836 ± 226

The proteinase activity increased in spinal cord and sciatic nerve tissues, between the 12th and 20th day after intoxication by either TOCP or DFP, the former having slightly greater effect. Activity did not change in brain tissue, and during the whole period of intoxication this tissue showed no sign of demyelination, although its cholinesterase activity was greatly inhibited by both poisons. Proteinase activity decreased in liver. Demyelination of spinal cord and sciatic nerves occurred with both poisons.

The increases in proteinase activity in spinal cord and sciatic nerve during intoxication by DFP and TOCP showed considerable differences from one another (Table 1). Although probably related to different degrees of demyelination, the differences were not sufficiently great to have caused histological changes which could be differentiated.

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Data on the changes in activity of sciatic nerve proteinase following DFP and TOCP poisoning are compared (Fig. 1) with those observed during myelin degeneration following transection (PORCELLATI and CURTI, 1960). These results show that, while the pattern of change for the two types of demyelination are similar, enzymic activity is quantitatively much higher after transection than after poisoning.

There was wide variation in the proteinase activity of liver after poisoning by organo-phosphorus compounds, compared with the other tissues examined (Table 1).

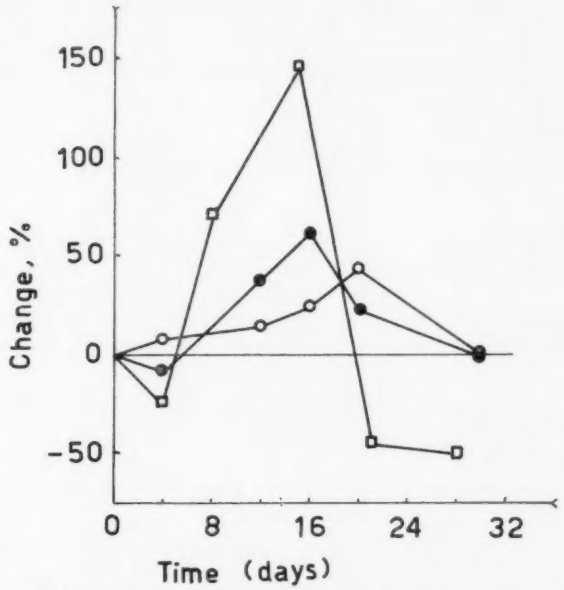

FIG. 1.—Proteinase activity of hen sciatic nerves during demyelination after TOCP or DFP poisoning, in comparison with that during myelin degeneration after transection (PORCELLATI and CURTI, 1960).
○ = DFP; ● = TOCP; □ = nerve section.

It is difficult to say whether the decrease in proteinase activity was due to direct inhibition of the enzymic system by the organo-phosphorus compounds, or to a general loss in activity of the liver cells caused by the debilitated state of the treated animals.

The increase in proteinase activity of nervous tissues seems to be an effect of demyelination rather than a cause of it, since in most cases the enzymic changes follow the first definite signs of true demyelination, and do not precede the structural and physiological nerve damage. Accordingly, the greatest increase in proteinase activity and the most evident stages of myelin degeneration were concomitant (16–20 days after poisoning). The increase did not seem to be related to proliferation of Schwann cells and macrophages, which occurs during the first days of demyelination. The quantitative difference between the increased proteinase activity during nerve demyelination by anticholinesterases and after transection is due probably to the weaker demyelination produced by the chemical agents.

It seems worth while extending these studies by examining proteinase activity

during intoxication by other anticholinesterases, which do not damage the spinal tracts and the nerve fibres, although they induce strong cholinesterase inhibition.

Proteins and their metabolism could be of importance in nerve degeneration, as they form structural elements of the myelin sheaths, bound in some way with lipids, in many parts of the nervous system. It seems probable then that these substances are involved in the process of demyelination and a careful study of their role in this connection is desirable.

SUMMARY

A study was made of proteinase activity in hen brain, spinal cord, sciatic nerve and liver after poisoning by tri-ortho-cresylphosphate (TOCP) or di-iso-propyl-fluorophosphonate (DFP). Proteinase activity in spinal cord and sciatic nerve increased as the period after administration increased, reaching a maximal value on the 16–20th day. It was unaffected in brain. The increase in activity in spinal cord and sciatic nerve appears to be an effect of the demyelination produced by the two anticholinesterases.

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